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The Journal of Biochemistry, Vol. 43, No. 3, 1956

STUDIES ON TAKA-AMYLASE A

V. CARBOXY-TERMINAL GROUP OF TAKA-AMYLASE A

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(Received for publication, February 6, 1956)

With the progress in the studies of the chemical structure of proteins a number of method for the identification of carboxy-terminal residues of proteins or peptides have been reported. In 1952, Akabori *et al* have reported a new method which is based on the following principle: proteins or peptides are treated with anhydrous hydrazine and converted to amino acid hydrazides and amino acids, the latter being derived only from carboxy ends (*1*). They have later applied Sanger's DNP-method to separate amino acids from amino acid hydrazides (*2*, *3*, *4*).

Other several investigators applied the hydrazinolysis method to some proteins obtaining the satisfactory results (*5*, *6*, *7*). On the other hand, it was pointed out by other workers that the results obtained by this method seemed to differ from those obtained with carboxypeptidase (*8*), and that the hydrazine is not always a suitable reagent for this purpose (*9*). However, the fundamental studies in hydrazinolysis in our laboratory eliminated the defect of this method and showed that the hydrazinolysis is a reliable method for the semiquantitative determination of *C*-terminal residues in proteins (*10*).

In our previous paper, it has been reported that taka-amylase A (TAA) has one *N*-terminal amino acid, alanine, and from its quantitative determination the minimum molecular weight of TAA has been calculated to be about 52,500 (*11*). The present report deals with the determination of *C*-terminal amino acids of TAA by the hydrazinolysis-chromatography method and the hydrazinolysis-dinitrophenylation-method.

MATERIALS AND METHODS

Taka-amylase A—Crystalline TAA prepared from "Takadiastase Sankyo" was recrystallized three times from aqueous acetone as described in the previous paper

(*12*). After washing with acetone the crystalline protein was dried in a vacuum desiccator.

Anhydrous Hydrazine—Hydrazine used in the following experiments was prepared by the method of Stähler (*13*). The purity was over 99 per cent.

Hydrazinolysis and Isovaleraldehyde Treatment—Fifty mg. of TAA were hydrazinolyzed with about 1 g. of anhydrous hydrazine at 100° avoiding the moisture, the time of hydrazinolysis being varied from 2 to 18 hours. After the removal of excess of hydrazine *in vacuo* over sulfuric acid, the residue was dissolved in 5 ml. of water, and treated with 0.3 ml. of isovaleraldehyde with occasional stirring, the pH of the solution being adjusted to about 7.0 by the addition of solid sodium bicarbonate. The amino acid hydrazides reacted with aldehyde forming resinous precipitate, which was filtered off and washed twice with 1 ml. of water. The combined solution of filtrate and washings were again treated with 0.3 ml. of isovaleraldehyde for the purpose of the complete removal of hydrazides. The solution was extracted with 5 ml. and then 3 ml. of ethyl acetate. The combined ethyl acetate layer was reextracted with 3 ml. of water. The water phase (total volume 10 ml.) contains amino acids derived from *C*-terminal residues and a part of isovaleral derivatives of aspartic and glutamic acid mono hydrazides.

Identification of Amino Acid by Column Chromatography—The water phase obtained by the isovaleraldehyde treatment of the hydrazinolyzate of TAA was evaporated in a vacuum desiccator. The residue was dissolved in an appropriate buffer solution and poured on the top of the ion exchange resin column.

The column and the buffer solution employed in this experiment and the other techniques, were same as reported by Moore and Stein. (*14, 15*).

Dinitrophenylation and Extraction of DNP-amino Acids—To the water phase (10 ml.) 0.3 g. of solid sodium bicarbonate and 20 ml. of ethanolic solution containing 0.3 ml. of fluorodinitrobenzene were added. After shaking the reaction mixture for 2 hours in the dark at room temperature, 50 ml. of water were added and then acidified with 2 *N* hydrochloric acid. The mixture was extracted with 30, 20, and 10 ml. of ethyl acetate. The ethyl acetate extracts were combined and extracted with 30, 30, 20 and 10 ml. of 2 per cent sodium bicarbonate solution, and the combined bicarbonate extracts were washed with 30, and 20 ml. of ethyl acetate. Most of dinitrophenyl (DNP)-amino acids and a part of isovaleral derivatives of DNP-aspartic and glutamic acid monohydrazides were transferred into sodium bicarbonate solution, while all of di-DNP-tyrosine together with a part of DNP-tryptophan and di-DNP-histidine were remained in the ethyl acetate layer. Therefore, the ethylacetate layers were combined and treated as described in the next item. The bicarbonate layer was acidified with 2 *N* hydrochloric acid and extracted with 30, 20 and 10 ml. of ethyl acetate. The final ethyl acetate solution was dried up *in vacuo*.

Separation of Di-DNP-tyrosine and DNP-tryptophan—The ethyl acetate layer mentioned above was evaporated *in vacuo* and the residue was dissolved in 50 ml. of ethyl ether, extracted with 30, 20 and 10 ml. of 2 per cent sodium bicarbonate solution, into the latter, di-DNP-tyrosine, DNP-tryptophan and di-DNP-histidine should be transferred. The combined aqueous extracts were acidified with 2 *N* hydrochloric acid and extracted

with 30, 20 and 10 ml. of ethyl ether, and the ether solution was dried up *in vacuo*. The residue should contain di-DNP-tyrosine and DNP-tryptophan, if these two amino acids are situated as *C*-terminals.

Chromatographic Identification and Quantitative Determination of C-Terminal Amino Acids—The separation and identification of DNP-amino acid were performed with silicagel chromatography described by Sanger (*16*) or Blackbarn (*17*). The each band of DNP-amino acid was cut off, eluted with ethyl acetate or ethyl alcohol, and dried up *in vacuo*, dissolved in 5 ml. of 1 per cent sodium bicarbonate solution, and determined colorimetrically using 430 mμ filter.

RESULTS AND DISCUSSION

Estimation of C-Terminal Amino Acids of TAA—Fifty mg. of TAA were hydrazinolyzed with 1 g. of anhydrous hydrazine for 5 hours and treated with isovaleraldehyde as mentioned above. After evaporation of the aqueous solution containing *C*-terminal amino acids of TAA, the residue was dissolved in citrate buffer (pH 3.42 or 5.0) and chromatographed by the method of Moore and Stein. (*14*, *15*) The results of chromatographic analyses are shown in Figs. 1 and 2.

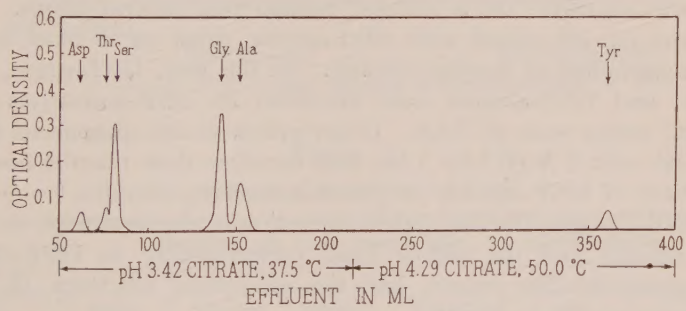


FIG. 1. Elution curve of a aldehyde treated hydrazinolyzate of 50 mg. of TAA (5 hrs. hydrazinolysis; determined by the ninhydrin method on a Dowex 50 column, 0.9 × 100 cm, for the acidic and neutral amino acids. The data given as optical densities (ordinate) without correction for the different color yields given by the individual amino acids. The abscissa shows the number of 1 ml. fraction collected.

Serine, glycine, alanine and tyrosine were calculated as 0.65, 0.78, 0.31 and 0.17 moles, respectively. Other ninhydrin positive peaks, A, B, C, D, E, and F as shown in Fig. 2, are supposed to be isovaleral derivatives of aspartic α-, β-, and glutamic α-, γ-hydrazides, or other

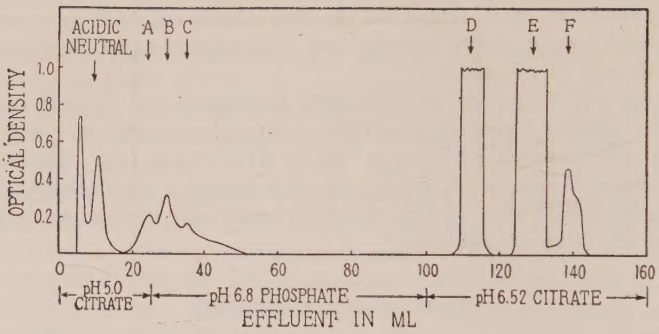


Fig. 2. Elution curve for the estimation of the basic amino acids on a 0.9×15 cm. column of Dowex 50. The aldehyde-treated hydrazinolyzate of 29 mg. of TAA was used.

water soluble isovaleral derivatives of some amino acid hydrazides.

In another series of experiments the aqueous solution obtained by the aldehyde treatment was dinitrophenylated and the DNP-amino acids were extracted as described above. The mixture of DNP-amino acids was separated on a silicagel column into several bands. Each band was cut off, eluted with ethyl acetate, dried up *in vacuo*, and rechromatographed on another column. In this way, DNP-serine, DNP-glycine, and DNP-alanine were identified as DNP-derivatives of *C*-terminal amino acids of TAA. Other yellow bands disappered by the hydrolysis with 2 *N* HCl for 3 hrs. and therefore these may be isovaleral derivatives of DNP-aspartic or glutamic mono-hydrazides.

DNP-Tyrosine fraction (ether extract) was also separated on a silicagel column and the yellow band corresponding to DNP-tyrosine was extracted. The extract had the adsorption spectrum of DNP-tyrosine as shown in Fig. 3.

For the purpose of confirming the absence of free amino acids or small peptides in TAA, dinitrophenylated TAA was extracted with ethyl acetate and the extract was subjected to chromatography, but none of DNP-amino acids or peptides could be found.

Quantitative Determination of C-Terminal Amino Acids—Fifty mg. of TAA were hydrazinolyzed for 2 to 18 hours and the liberated *C*-terminal amino acids were quantitatively determined by the DNP-technique as mentioned above. The results are shown in Table I.

When the hydrazine solution of TAA was dried up without heating *in vacuo* at room temperature, 0.3 moles of serine and 0.1 moles of

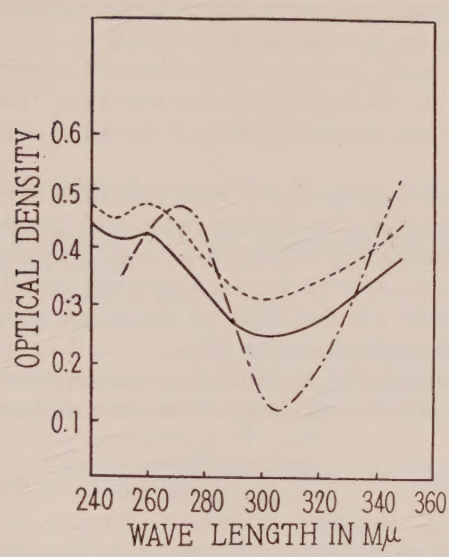


FIG 3. Spectral absorption curves of DNP-amino acids in 1 per cent $NaHCO_3$.

- - - - - - DNP-tyrosine, — - — - — - — DNP-tryptophan,
———— DNP-tyrosine fraction of hydrazinolyzate of TAA.

TABLE I

C-Terminal Amino Acids of TAA

Time of hydrazinolysis	Residues per mole protein (mol. wt. 52,500) found					
	Serine	Glycine	Alanine	Tyrosine	Aspartic acid fraction	Threonine fraction
hrs.						
2	0.6	0.4	trace			
5	0.7	0.8	0.3	0.17	0.1	0.1
10	0.8	0.8	0.5			
12	0.7	1.2	0.7	0.2	1.4	0.6
15	0.6	0.9	0.8			
18	0.8	1.0	0.7	0.6	1.1	0.6

glycine were detected as *C*-terminal residues. From the data in Table I it is clear that each one glycine and one serine are really the *C*-terminals of this protein. However, as alanine were liberated only 0.3 and

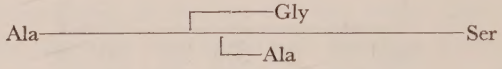
0.5 moles by hydrazinolysis for 5 and 10 hours respectively, there may be some doubt on its presence at *C*-terminal.

On the other hand, the rate of liberation of *C*-terminal alanine from L-leucyl-L-alanine by the hydrazinolysis is slow as in TAA (*10*), it is probable that *C*-terminal alanine in TAA is bound to a hardly hydrazinolysable bond.

By a prolonged hydrazinolysis, some quantities of tyrosine, aspartic acid and threonine are detected as free amino acid, but they are probably produced by the secondary hydrolysis of their hydrazides.

In our previous paper, it has been reported that TAA has one *N*-terminal amino acid, alanine, and its minimum molecular weight is 52,500 per one *N*-terminal residue (*11*).

From the experimental results and considerations described above, the author proposed the following schematic structure for TAA.



Although the author has no information on the mode of branching, it is probable that glutamic γ- or aspartic β- bond is involved in the branching structure of TAA.

As for one example of such a structure, pepsin has been reported to have one *N*-terminal, isoleucine (*18*) and two *C*-terminals, alanine, the latter being determined by the reduction method using lithium aluminium hydride (*19*).

The carboxypeptidase method hitherto widely used cannot give any reliable information about the number of *C*-terminals of proteins, when they have more than two *C*-terminals. On the contrary, hydrazinolysis method gives more reliable results in such cases as shown in this experiment.

SUMMARY

The hydrazinolysis method has been applied to the identification of *C*-terminals of taka-amylase A and serine, glycine and alanine were found as *C*-terminal amino acids. From the quantitative studies, it has been suggested that taka-amylase A may have three *C*-terminals against one *N*-terminal.

The author wishes to express his gratitude to Professor S. Akabori for his kind guidance, and to Dr. K. Ohno for his helpful advice through this investigation, and

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CHEMICAL STUDIES ON THE ORGAN SPECIFICITY

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(Received for publication, February 13, 1956)

In 1903, Uhlenhuth (*1*) reported that the antiserum obtained by immunizing rabbits with bovine eye lenses reacted not only with bovine lenses but also with those of animals of other species. Since this discovery, the fact was confirmed by many investigators and it became clear that the brain, adrenal glands, and hypophysis of various animals also possess the same as an eyelens in serological reaction.

Many investigators have reported on the chemical nature of the substances, which are contained in these tissues and react specifically. Yamada (*2*) observed that sphingomyeline and cerebroside obtained from pyridine extract of brain tissues indicated a phenomenon of organ specificity. Wood and Burly (*3*) experimented with the eyelens and concluded that the specific substances in the eyelens were α-, and β-crystallin. S. Fujimura (*4*, *5*) studied the same problem with brain tissues and reported that the specific substance could be detected in crude phospholipid fraction of the brain tissues, but when the phospholipid was purified its activity disappeared, while an impurity accompanied with it indicated the serological activity. This active substance could be considered to be a protein.

The author attempted to clear the chemical nature of the organ-specific substance in eyelens and to what chemical structure of it is due the phenomenon of the organspecificity.

EXPERIMENTAL AND RESULT

Method of Immunization and Serological Reaction

Eye lenses of ox, horse, or pig were freed from connective tissues and washed carefully with physiological saline solution. They were homogenized with a glass homogenizer, extracted with 30 parts of physiological saline solution for two hours and then filtered. Rabbits were immunized by the administration of 3 ml. of the filtrate every

other day for 4 weeks. On the 7th day after the last injection, the blood was drawn and used for the experiments.

As the serological reaction, the complement fixation reaction was used. The antiserum was inactivated by heating at 56° for 30 minutes and diluted ten times with physiological saline solution. As the complement, guineapig serum was always used. An amount of complement, which is sufficient to hemolyse 0.5 ml. of 3 per cent suspension of bovine red corpuscles in the presence of 5 units of hemolysins, was taken as one unit.

In a series of test tubes, 0.5 ml. of 10 per cent antiserum, an adequate amount of testing substance from the eye lens and increasing amount of complement of 2, 4, 6, and 8 units were placed and mixed well. After allowing to stand for one hour at 37°, 0.5 ml. of the suspension of sensitized corpuscles was added to each of the tubes and incubated for one hour at 37°. At the end of this period degrees of hemolysis were observed.

In the tables, cases where no hemolysis occurs are indicated as (—), and complete hemolysis as (#), while for intermediate cases signes (±), (+), and (#) are used.

In this work, the substances with which rabbits were immunized were termed as antibody producer, and those which were used to test the formation of antibodies in serological reaction as antibody detector. The results are shown in Table I.

The results of the complement fixation reactions with the anti-oxlens serum and extracts of other organs of ox are shown in Table II.

As indicated in Tables I and II antiserum, by immunization of rabbits with ox-lens the antiserum, which reacted with eyelens specifically, was obtained, and these sera were used in the following experiments.

Fractionation of the organ specific substance from eyelens.

Homogenates of ox, horse, or pig eyelens were successively extracted throughly with acetone, ether and pyridine. Each extract was concentrated to dryness in a low pressure in a slow current of carbon dioxide at a temperature not exceeding 40°, and dissolved in alcohol.

The residue insoluble in acetone, ether, and pyridine was finally extracted with physiological saline solution.

Activties as an antibody detector against the eyelens antiserum tested with these extracts. Each of the alcoholic solution was mixed with one per cent alcoholic solution of cholesterol in a ratio of 3 :1 and emulsified by diluting to 30 volumes with physiological saline solution. In an interval of 30 to 60 minutes after the emulsification they were used

TABLE I

Complement Fixation Reaction with Anti-oxlens Serum and Extract of Eyelens of Ox

No. of rabbit	Antibody producer	Antibody detector	Units of complement						Control test	
			2	4	6	8	10	12	Serum + Compl.	Antigen + Compl.
105	Ox lens	Ox lens	—	—	—	—	±	##	##	##
106	,,	,,	—	—	—	—	—	±	##	##
108	,,	,,	—	—	—	—	±	##	##	##
105	,,	Pig lens	—	##	##	##	##	##	##	##
106	,,	,,	—	—	—	—	##	##	##	##
108	,,	,,	—	—	—	±	##	##	##	##
105	,,	Guinea pig lens	±	##	##	##	##	##	##	##
106	,,	,,	—	—	—	—	±	##	##	##
108	,,	,,	—	—	—	—	±	##	##	##
105	,,	Rabbit lens	—	—	+	##	##	##	##	##
106	,,	,,	—	—	+	##	##	##	##	##
108	,,	,,	—	—	##	##	##	##	##	##
106	,,	Horse lens	—	—	—	±	##	##	##	##
108	,,	,,	—	—	±	##	##	##	##	##

as an antibody detector in the sorological reactions.

The results are illustrated in Table III.

As can be seen from the results, acetone or ether extract of eyelens of ox or horse was completely free from the specific substance and although a trace of it was detected in the warm pyridine extract it disappeared when the extract was reextracted with warm pyridine after concentrating to dryness and washing with acetone. On the other hand, the saline extract of the residue, which was freed previously from substances soluble in acetone, ether, and warm pyridine, was active as an

TABLE II

Complement Fixation Reaction with the Anti-Oxlens Serum and Extracts of Other Organs of Ox as the Antibody-detector

No. of rabbit	Antibody producer	Antibody detector	Units of compl.					Control test	
			2	4	6	8	10	Serum + Comple.	Antigen + Compl.
106	Ox lens	Liver extract	##	##	##	##	##	##	##
108	"	"	##	##	##	##	##	##	##
106	"	Heart extract	##	##	##			##	##
108	"	"	##	##	##			##	##
106	"	Lung extract	##	##	##			##	##
108	"	"	##	##	##			##	##
106	"	Spleen extract	##	##	##			##	##
108	"	"	##	##	##			##	##
106	"	Brain extract	##	##	##			##	##
108	"	"	##	##	##			##	##
106	"	Kidney extract	##	##	##			##	##
108	"	"	##	##	##			##	##
106	"	Muscle extract	##	##	##			##	##
108	"	"	##	##	##			##	##

antibody detector. It can be seen, therefore, that the specific substance in eyelens is soluble in saline solution but not in organic solvents such as acetone, ether, or pyridine.

Fractionation of Proteins from the Eyelens

From the previous experiments, it was considered that the specific substance in the eyelens may be a substance other than lipids, and in the present experiment, the fractionation of protein in eyelens was attempted and experiments were performed to clarify whether or not the protein the eyelens carried the activity as the antibody detector.

TABLE III

Complement Fixation Reaction with the Oxlens Antiserum and Certain Fractions of Eyelens of Ox, horse or Pig

No. of rabbit	Antibody producer	Antibody	Units of complement					Control test	
			2	4	6	8	10	Antiserum +Compl.	Antigen +Compl.
105	Ox eye lens	Acetone extract (ox)	++	++	++	++		++	++
106	"	"	++	++	++			++	++
105	"	Ether extract (ox)	++	++	++			++	++
106	"	"	±	++	++			++	++
105	"	Pyridine extract (ox)	−	++	++			++	++
106	"	"	++	++	++			++	++
105	"	Saline extract (ox)	−	−	−	++	++	++	++
106	"	"	−	−	±	++	++	++	++
105	"	Acetone extract (horse)	++	++	++	++		++	++
106	"	"	++	++	++	++		++	++
105	"	Ether extract (horse)	++	++	++	++		++	++
106	"	"	++	++	++	++		++	++
105	"	Pyridine extract (horse)	±	++	++	++		++	++
106	"	"	−	++	++	++		++	++
105	"	Saline extract (horse)	−	−	±	++		++	++
106	"	"	−	−	−	++		++	++

The homogenate of eyelens of ox or horse was successively extracted with acetone and ether, and dried. From this residue, albumoid, and α-, β-, and γ- crystallins, were fractionated according to the method

described by A. C. Kraus (*6*). These fractions were used as an antibody detector in complement fixation reaction and results obtained are listed in Table IV.

TABLE IV

Complement Fixation Reaction with the Eyelens Antiserum and Several Protein Fractions of the Eyelens

No. of rabbit	Antibody producer	Antibody detector	Units of complement					Control test	
			2	4	6	8	10	Antiserum +Compl.	Antigen +Compl.
105	Ox lens	Albumoid (ox)	+	##	##	##		##	##
106	"	"	−	+	##	##		##	##
105	"	α-crystallin (ox)	−	−	−	##		##	##
106	"	"	−	−	±	##		##	##
105	"	β-crystallin (ox)	−	−	##	##		##	##
106	"	"	−	±	##	##		##	##
105	"	γ-crystallin (ox)	##	##	##			##	##
106	"	"	##	##	##			##	##
105	"	Albumoid (horse)	−	±	##	##		##	##
106	"	"	+	##	##	##		##	##
105	"	α-crystallin (horse)	−	−	±	##	##	##	##
106	"	"	−	−	##	##	##	##	##
105	"	β-crystallin (horse)	−	−	##	##	##	##	##
106	"	"	−	−	##	##	##	##	##
105	"	γ-crystallin (horse)	±	##	##	##	##	##	##
106	"	"	##	##	##	##		##	##

We know from these results that among the protein fractions of ox and horse eyelens, albumoid, and α- and β-crystallin are active as an antibody detector against the eyelens antiserum and γ-crystallin is almost inactive.

TABLE V(A)

Complement Fixation Reaction with the Eyelens Antiserum and the Partial Hydrolysates of Crystallin of Eyelens

(1 *N* sulfuric acid used for the hydrolysis)

No. of rabbit	Antibody producer	Antibody detector (duration of hydrolysis)	Units of compl.				Control test	
			2	4	6	8	Antiserum + Compl.	Antigen + Compl.
105	Ox lens	0.5 *hrs.* (ox)	##	##			##	##
106	"	"	##	##			##	##
108	"	"	##	##			##	##
105	"	1 (ox)	##	##			##	##
106	"	"	##	##			##	##
108	"	"	##	##			##	##
105	"	0.5 (horse)	##	##			##	##
106	"	"	##	##			##	##
108	"	"	##	##			##	##
105	"	1 (horse)	##	##			##	##
106	"	"	##	##			##	##
108	"	"	##	##			##	##

Relation between the Organ Specificity and Partial Hydrolysis of Eyelens Proteins.

From the above experiments, it is seen that the protein fractions, especially α- and β-crystallin, of eyelens of ox and horse markedly indicated the activities as an antibody detector against the eyelens antiserum. It can be supposed that the organ specificity is due to the fact that the molecules of these proteins in eyelens of various animals involve some groups which react commonly in serological reaction.

It is a well-known fact that Atoxyl (*p*-aminobenzenearsinic acid) does not react with the antiserum obtained by immunizing rabbits with atoxylazoprotein, but inhibits the serological reaction between

the latter and the corresponding antiserum, probably by combining with the antibodies at the active groups in them.

Considering that if the protein of the eyelens is hydrolyzed incompletely to some extent, it may lose the activity as an antibody detector but be able to inhibit the serological reaction between the eyelens protein and the corresponding antibodies, the crystallin fraction of the eyelens of ox and horse was hydrolyzed with sulfuric acid and the activities as an antibody detector and an inhibitor were tested on the hydrolysates from time to time during hydrolysis as described below.

The crystallin fractions of eyelens of ox or horse were suspended in 1 or 5 N sulfuric acid in a final concentration of 10 per cent, and boiled in a flask with a condenser. During the process of hydrolysis a small portion of the content was taken out neutralyzed with barium hydroxide to remove the sulfate as a precipitate of barium sulfate, and the supernatant liquid was separated by centrifugation to be used for the test.

The results are indicated in Table V.

As can be seen from Table V (A) and (B) the crystallin of eyelens of ox or horse lost the activity as an antibody detector against the eyelens antiserum by hydrolysis with 1 or 5 N sulfuric acid for 0.5 or 0.3 hours, respectively.

In order to find the duration of hydrolysis necessary for the eyelens crystallin to lose the activity of inhibiting the serological reaction between eyelens protein and the corresponding antiserum, the following experiments were performed.

In a series of test tubes 0.5 ml. of 10 per cent antiserum and the adequate amount of the incomplete hydrolysates of eyelens crystallin were mixed and were incubated for one hour at 37°. After the incubation suspension of ox-eyelens homogenate and complement in an increasing amount of 2, 4, and 6 units were added and incubated again for 1 hour at 37°. At the end of this period, 0.5 ml. of 3 per cent suspension of sensitized corpuscles was added to each tube and after an incubation for one hour at the same condition, degree of hemolysis was observed. At the same time biuret reaction and trichloroacetic and sulfosalicylic acid tests were performed on the hydrolysates.

The results are shown in Table VI.

From these results, it is seen that the inhibitory activity of the eyelens crystallin of ox or horse diminishes completely by hydrolysis with 5 N sulfuric acid for one hour. In other words, it may be said that partial hydrolysis effects decomposition of certain groups in the molecules

TABLE V(B)

Complement Fixation Reaction with the Eyelens Antiserum and the Partial Hydrolysates of Crystallin of Eyelens

(5 *N* sulfuric acid used for the hydrolysis)

No. of rabbit	Antibody producer	Antibody detector (Duration of hydrolysis)	Units of compl.				Control test	
			2	4	6	8	Antiserum + Compl.	Antigen + Compl.
105	Ox lens	*hrs.* 1/3 (ox)	##	##	##		##	##
106	"	"	##	##	##		##	
105	"	1/2 (ox)	##				##	##
106	"	"	##					
105	"	2/3 (ox)	##					##
106	"	"	##					
105	"	1 (ox)	##	##				##
106	"	"	##	##				
105	"	1/3 (horse)	##	##				##
106	"	"	##	##				
105	"	1/2 (horse)	##	##				##
106	"	"	##	##				
105	"	2/3 (horse)	##	##				##
106	"	"	##	##				
105	"	1 (horse)	##	##				##
106	"	"	##	##				

of eyelens crystallin, with which it can combine with the corresponding antibodies.

Chemical Nature of the Partial Hydrolysates which is Active as the Inhibitor.

From above experiments, it became clear that a simple compound, which inhibits the serological reaction between the eyelens crystallin and

Table VI

Inhibitory Activities of the Incomplete Hydrolysates of the Eyelens Crystallin and Protein Reactions

($5\,N$ H_2SO_4)

No. of rabbit	Antibody producer	Inhibitor (duration of hydrolysis)	Biuret reaction	Precipitation		Units of compl.			Control test	
				Trichlor acetic acid	Sulfosalicylic acid	2	4	6	Antiserum + Compl.	Antigen + Compl.
105	Ox lens	*hrs.* 1/3 (ox)	++	+++	±	+++	+++	+++	+++	+++
"	"	½ (ox)	++	+++	±	+++	+++	+++	+++	+++
"	"	2/3 (ox)	++	++	−	+++	+++		+++	+++
"	"	1 (ox)	++	+	−	++	+++		+++	+++
"	"	1½ (ox)	+	−	−	++	++		+++	+++
"	"	2 (ox)	+	−	−	+	+++		+++	+++
"	"	2½ (ox)	±	−	−	−	+++		+++	+++
"	"	3 (ox)	±	−	−	−	+++		+++	+++
"	"	5 (ox)	±	−	−	−	+++		+++	+++
"	"	1/3 (horse)	+++	+++	±	+++	+++		+++	+++
"	"	2/3 (horse)	+++	+++	±	+++	+++	+++	+++	+++
"	"	1 (horse)	++	+	−	+++	+++	+++	+++	+++
"	"	1½ (horse)	+	+	−	++	+++	+++	+++	+++
"	"	2 (horse)	+	−	−	++	+++	+++	+++	+++
"	"	2½ (horse)	±	−	−	+	+++	+++	+++	+++
"	"	3 (horse)	±	−	−	−	++	+++	+++	+++
"	"	5 (horse)	±	−	−	−	+++	+++	+++	+++

the corresponding antibodies and combining with the antibodies, is obtained by hydrolysis of the eyelens crystallin with $5\,N$ sulfuric acid

for 1 hour. These partial hydrolysates indicate a positive biuret reaction and are precipitated by trichloroacetic acid, but not by sulfosalicylic acid.

These active substances from ox or horse eyelens crystallin react not only with the corresponding antibodies, but also with those which are produced by immunization of rabbits with eyelens of animals of other species. It can, therefore be supposed that they are substances which are serologically common to each other.

To find out some chemical nature of these substances the following investigations were performed:

A) Dialysis—0.5 ml. of the partial hydrolysate of ox or horse eyelens crystallins was dialysed in a collodium membrane against running water for 40 hours. At the end of this period the inhibitory activity was tested on the content of the membrane.

The results are indicated in Table VII.

TABLE VII

Activity of the Dialysate of the Active Partial Hydrolysate of Ox or Horse Eyelens Crystallin as An Inhibitor

No. of rabbit	Antibody producer	Inhibitor (Dialysate)	Antibody detector	Units of compl.			Control test	
				2	4	6	Antiserum + compl.	Antigen + compl.
105	Ox lens	Partial hydrolysate of ox lens	Ox lens	−	±	##	##	##
106	"	"	"	−	+	##		
105	"	Partial hydrolysate of horse lens	"	−	−	##		
106	"	"	"	−	−	##		

As can be seen from these results, the activity of the partial hydrolysate of ox or horse lens crystallins as an inhibitor was lost by dialysis, and it is seen that the active substances are dialysable.

B) Adsorption—To the partial hydrolysates activated charcoal was added to a concentration of one per cent at pH 4.0, 5.0, 7.0, or 9.0, and let stand at 0° for one hour. After the standing each of the

filtrates was neutralyzed and the activity as an inhibitor was tested.

The results are illustrated in Table VIII.

TABLE VIII

Effect of Treatment with Activated Charcoal upon the Activity of the Partial Hydrolysate as An Inhibitor

No. of rabbit	Antibody producer	Inhibitor (partial hydrolysate treated at)	Antibody detector	Units of compl.			Control test	
				2	4	6	Antiserum + compl.	Antigen + compl.
105	Ox lens	pH 4.0 (ox)	Ox lens	−	−	##	##	##
"	"	pH 5.0 (ox)	"	##	##	##	##	##
"	"	pH 7.0 (ox)	"	##	##	##	##	##
"	"	pH 9.0 (ox)	"	##	##	##	##	##
106	"	pH 4.0 (horse)	"	−	##	##	##	##
"	"	pH 5.0 (horse)	"	±	##	##	##	##
"	"	pH 7.0 (horse)	"	##	##	##	##	##
"	"	pH 9.0 (horse)	"	##	##	##	##	##

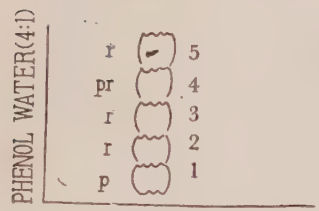
The results of the experiments indicate that the active substances were completely adsorbed by charcoal at pH 5.0, 7.0, and 9.0 but not at pH 4.0. The active substances adsorbed by charcoal could not be reextracted with physiological saline solution in acid nor alkaline reactions.

C) Paper-chromatography—The partial hydrolysate of the eyelens crystallins from ox or horse was examined by a paper chromatography of one-and two dimensions. For the identification of the inhibitory substances from the spots, phenol-water (4 :1) and n butanol- acetic acid- water (4 :1 :2) were used as the 1st and 2nd developers, respectively. In the chromatogram five twin spots with Rf values of 0.17, 0.08 ; 0.25, 0.21 ; 0.31, 0.35 ; 0.57, 0.56 ; 0.77 ; 0.76 were detected. The spots were colored by 0.1 per cent ninhydrin butanol solution and the first spot colored purple, the second and third reddish, the fourth purple

reddish, and the fifth reddish. Further, the partial hydrolysate was, developed with butanol- acetic acid-water on paper chromatogram and eleven or more spots were obtained. The results are illustrated in Figs. 1 and 2.

FIG. 1. One-and two-dimensional paper chromatograms of the partial hydrolysates.

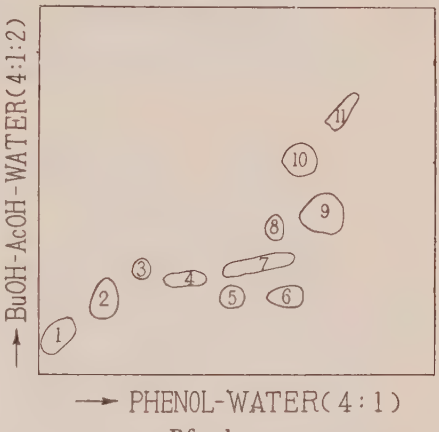
P: purple
pr: purple reddish
r: reddish



Rf values

Spot No.	Horse lens	Ox lens
1	0.17	0.08
2	0.25	0.21
3	0.31	0.35
4	0.57	0.56
5	0.77	0.76

FIG. 2



Rf values

Spot No.	Phenol-water (4:1)	Butanol-acetic acid-water (4:1:2)
1	0.0	0.13
2	0.09	0.35
3	0.17	0.36
4	0.31	0.33
5	0.58	0.27
6	0.65	0.32
7	0.52	0.39
8	0.67	0.50
9	0.77	0.50
10	0.70	0.60
11	0.77	0.79

Isolation of Inhibitory Substance by A Column Chromatogram.

Glass tubes, 270～300 mm. in length and 19 mm. in diameter, were filled with activated alumina, 40 to 50 ml. of the partial hydrolysate of eyelens crystallins concentratcd to 1/5 the original volume were poured in each glass tube, and allowed to develope.

The alumina was taken out in several portions, avoiding both ends of the tube and extracted with a small amount of 0.15 *M* saline solution at 60°. The inhibitory activity was tested on each extract and reaults show in Tables IX and X were obtained.

TABLE IX

Inhibitory Activity of the Extract of Column Chromatogram from the Partial Hydrolyates of Ox Lens Crystallin

No. of rabbit	Antibody producer	Inhibitor (extract of column chromatogram distant from upper edge)	Biuret reaction	Antibody detector	Units of complement		
					2	4	6
106	Ox lens	0— 30 mm.	++	Ox lens	−	++	++
„	„	30— 60 „	+	„	±	++	++
„	„	60— 90 „	+	„	++	++	++
„	„	90—120 „	+	„	−	++	++
„	„	120—150 „	±	„	−	++	++
„	„	150—180 „	+	„	−	++	++
„	„	180—210 „	+	„	−	++	++
„	„	210—240 „	+	„	−	++	++
„	„	240—270 „	+	„	−	++	++

As illustrated in the foregoing tables, extracts of the portion 60 to 90 mm. from the upper edge of the alumina column of ox crystallin, and the portion 240 to 270 mm. of horse crystallin contain the inhibitory substances.

These active extracts were hydrolysed by boiling with 35 per cent sulfuric acid until biuret reaction disappears completely but xantho-protein, ninhydrine and Millon's test remain positive, developed on

TABLE X

Inhibitory Activity of the Extract of Column Chromatogram from the Active Patial Hydrolysate of Horse Lens Crystallin

No. of rabbit	Antibody producer	Inhibitor (extract of column chromatogram distant from upper edge)	Biuret reaction	Antibody detector	Units of complement		
					2	4	6
106	Ox lens	0— 30 mm.	++	Ox lens	—	+++	+++
"	"	30— 60 "	++	"	—	+++	+++
"	"	60— 90 "	+	"	—	+++	+++
"	"	90—120 "	+	"	—	+++	+++
"	"	120—150 "	+	"	—	+++	+++
"	"	150—180 "	+	"	—	+++	+++
"	"	180—210 "	+	"	+	+++	+++
"	"	210—240 "	+	"	+	+++	+++
"	"	240—270 "	+	"	++	+++	+++

TABLE XI

Amino Acids in the Hydrolysates of Extracts from Column Chromatogram

Ox lens	Horse lens	Pig lens
Aspartic acid	Serine	Alanine
Glutamic acid	Glutamic acid	Glutamic acid
Alanine	Alanine	Cystine
Tyrosine	Histidine	Histidine
Serine	Isoleucine	Serine
Histidine	Tyrosine	Tyrosine
Glycine		

a two-dimensional paper chromatogram with butanolacetic acid-water

(4 : 1 : 2) and phenol-water (4 : 1), and were colored with 0.1 per cent ninhydrine solution the following amino acids were dectected in each hydrolysate.

From this result the inhibiting substances, which were obtained by partial hydrolysation of ox, horse and pig eyelens crystallins, consist of relatively few kinds of amino acids common to all.

From the foregoing experiments, it became clear that the organ specificity of the eyelens was due to a polypeptide residue which is contained in the molecule of the eyelens crystallin and consists of a few kinds of amino acids of common to all.

In the next experiments, some chemical treatment was applied on the partial hydrolysate of the eyelens crystallin and its effect upon the activity as an inhibitor was observed.

A) Effect of Coupling with Diazobenzene-sulfanylic Acid—Diazobenzene-sulfanylic acid was coupled with the partial hydrolysate and its effect upon the activity was examined. Diazobenzenesulfanylic acid was prepared as follows.

10 g. of sulfanylic acid was diluted with 15 ml. of water, to which 10 ml. of concentrated hydrochloric acid was added to it. 10 ml. of 50 per cent sodium nitrite solution was added to it drop by drop, under cooling with ice water. White crystallines of diazobenzene-sulfanylic acid were obtained.

To 5 ml. of 0.2 per cent solution of this acid 5 ml. of 0.5 per cent solution of α- or β-crystallins and 1 ml. of 1 N sodium carbonate solution were added and let stand for 30 minutes. at 2°. Then 0.2 ml. of 0.2 per cent diazobenzene-sulfanylic acid and 0.5 ml. of 1 N sodium carbonate solution were added to it the mixture was and incubated for 30 minutes at 20°. This mixture was neutralized with hydrochloric acid and dialyzed in a collodium membrane.

In this procedure, tyrosine or histidine residue in the molecule of the partial hydrolysate of eyelens crystallin couples with diazobenzene-sulfanylic acid. The acitvity of substance thus obtained as an inhibitor was tested. The results are listed in Tables XII and XIII.

B) Effect of Esterification of the Partial Hydrolysate of Eyelens Crystallin upon Its Activity as An Inhibitor—The partial hydrolysate of the eyelens crystallin was dissolved in absolute alcohol and was saturated with chlorine by bubbling dried chlorine gas through the solution for 10 mins. at 50°. By this treatment free carboxyl groups which may be contained in the molecules of the compound were esterified with ethyl groups.

TABLE XII

Effect of Coupling of Diazobenzene-sulfanylic acid with the Active Partial Hydrolysate of Lens Crystallin upon Its Activity as An Inhibitor

No. of rabbit	Antibody producer	Inhibitor (diazotized protein of)	Antibody detector	Units of compl.		
				2	4	6
105	Ox lens	Ox lens crystallin	Ox lens	±	##	##
106	"	"	"	±	##	##
105	"	Horse lens crystallin	"	−	##	##
106	"	"	"	±	##	##

TABLE XIII

Complement Fixation Reaction with the Diazotized Protein Fraction and the Eyelens Antiserum

No. of rabbit	Antibody producer	Antibody detector (diazotized protein of)	Units of compl.			Control test	
			2	4	6	Antiserum + compl.	Antigen + compl.
105	Ox lens	Ox lens crystallin	##	##	##	##	##
106	"	"	##	##	##	##	
105	"	Horse lens crystallin	##	##	##	##	##
106	"	"	##	##	##	##	

After driving off the excess of chlorine by bubbling carbon dioxide gas through the solution, the activity of the solution as an inhibitor was tested. The results are shown in Table XIV.

From these results, it is concluded that tyrosine and histidine residues and carboxyl groups in the molecules of the active substance are all necessary for the activity of the partial hydrolysate as an inhibitor.

SUMMARY

Chemical studies on the organ specificity were performed using

TABLE XIV

Effect of Esterification of Carboxyl Groups in the Partial Hydrolysate of Eyelens Crystallin upon Its Activity as An Inhibitor

No. of rabbit	Antibody producer	Inhibitor (esterified protein of)	Antibody detector	Units of compl.			Control test	
				2	4	6	Antiserum + compl.	Antigen + compl.
105	Ox lens	Ox lens hydrolysate	Ox lens	−	##	##	##	##
106	"	"	"	−	±	##	##	##
105	"	Horse lens hydrolysate	"	−	+	##	##	##
106	"	"	"	−	+	##	##	##

eyelens crystallin of ox, horse, and pig and the following results were obtained:

1. Sphingomyelin, cerebroside, and other lipids contained in the eyelens are not responsible for the organ specificity.

2. α- and β-Crystallins of eyelens react specifically, but γ-crystallin does not.

3. The ability of the crystallins to react with the eyelens antiserum is lost by hydrolysis with 1 N or 5 N sulfuric acid for 0.5 or 0.3 hours, but the activity of inhibiting the serological reaction between lens crystallin and the corresponding antibodies remains intact when it was hydrolysed with 5 N sulfuric acid for one hour.

4. These partial hydrolysates scarecely show biret reaction but are not precipitated by sulfosalicylic or trichloroacetic acid, and can be dialyzed throughly collodium membran.

5. When the partial hydrolysate is coupled with diazobenzene-sulfanylic acid or the free carboxyl groups in it are esterified with ethyl groups the activities are destroyed.

6. The active substances in the partial hydrolysates of the eyelens crystallins of ox or horse can be isolated by column chromatography, and by the complete hydrolysation, they liberate several amino acids common to all, other such as serine, histidine, glutamic acid, alanine, and tyrosine.

From these facts, it may be considered that the specific substances in eyelens of animals are α- and β-crystallins and the chemical structures

which is responsible for organ specificity are groups of comparatively small polypeptide residues.

The author wishes to express his most sincere thanks to prof. S. Fujimura for his kind advices through this research.

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BIOSYNTHESIS OF THREONINE FROM HOMOSERINE

V. NATURE OF AN INTERMEDIARY PRODUCT[1]

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In the previous studies of this series (*1*, *2*), the over-all reaction of threonine synthesis from homoserine has been shown to be catalyzed by two enzymes prepared from acetone-dried yeast. From the studies on the order of the reaction sequence, the reaction mechanism has been formulated as in the following scheme. In the first step (Reaction 1, a), homoserine was converted to an intermediary product by *Enzyme I* in the presence of adenosinetriphosphate (ATP) and Mg^{++}, and in the second step (Reaction 1, b), the intermediate was transformed into threonine.

$$\text{(1, a)} \quad \text{Homoserine} + \text{ATP} \xrightarrow[Mg^{++},\ \textit{Enzyme I}]{} \text{intermediate}$$

$$\text{(1, b)} \quad \text{Intermediate} \xrightarrow[\textit{Enzyme II}]{} \text{threonine}$$

The present paper concerned with the isolation and the identification of the intermediate and with the results obtained in studies of the mechanism of the postulated reactions.

EXPERIMENTAL

Materials—DL-Homoserine and ATP were prepared as described earlier (*3*). *Enzyme I* and *Enzyme II* were prepared from acetone-dried baker's yeast by ammonium sulfate fractionation as described in the previous paper (*2*). P^{32}-labeled ATP was prepared by the method of Hems and Bartley (*4*), except that mitochondria of rat liver instead of homogenates of sheep heart was employed according to Ogata[2] P^{32}-labeled ATP having a specific activity of 3.10×10^4 c.p.m. per micromole of acid-labile phosphate was diluted before use with non-radioactive ATP to give

1) This work was supported by a Grant in Aid for Fundamental Scientific Research from the Ministry of Education.

2) Private correspondence.

a specific activity of 1.77×10^3 c.p.m. per micromole of labile phosphate.

Analytical Methods—The method used to determine threonine was described earlier (*3*). Inorgnic phosphate was determined by the method of Allen (*5*). Acid-labile phosphate was estimated as inorganic phosphate after splitting with *N* HCl at 100° for 10 minutes. Homoserine was determined spectrophotometrically after converted to dinitrophenyl derivative, applying the method of Mills (*6*) with slight modifications. Namely, aliquots of the deproteinized reaction mixture were treated with 2,4-dinitrofluorobenzene, condensed if necessary, and then subjected to paper chromatography in butanol saturated with 4 per cent ammonia. The spot corresponding to DNP-homoserine was eluted with 4 per cent sodium bicarbonate and the optical density of the eluate was determined at 360 mμ in a Beckman spectrophotometer.

O-Phosphohomoserine was determined by measuring the radioactivity of P^{32} transferred from P^{32}-labeled ATP. After it was separated from other radioactive compounds by paper electrophoresis at pH 3.8, the area of *O*-phosphohomoserine was located with ninhydrine, cut out, and eluted with water until the radioactivity left on the paper was negligible. The eluate was dried on a water bath and taken up in adequate volume of water. The radioactivity of aliquots was measured and expressed as micromoles of *O*-phosphohomoserine calculating from the specific activity 1.86×10^3 c.p.m. per micro mole of organic phosphorus of isolated *o*-phosphohomoserine.

Preparation of DNP-O-phosphohomoserine—DNP-DL-homoserine was prepared from 0.5 g. of DL-homoserine by the method of Mills (*6*), and recrystallyzed from alcohol by the addition of water; yield 0.7 g., m.p. 183°.

To a cooled solution of 0.2 g. of DNP-DL-homoserine in 2 ml. of pyridine and 5 ml. of water, 1.0 g. of $POCl_3$ dissolved in 3 g. of chloroform was added dropwise with continuous shaking and cooling in an ice bath. After the addition was completed, the mixture was kept at room temperature for 2 hours and then evaporated to dryness *in vacuo*. The residue was taken up in 2 *N* HCl and insoluble material was filtered off. The yellow filtrate was extracted with ether in a Soxhlet's extractor to remove the remaining DNP-DL-homoserine. The aqueous phase containing DNP-*O*-phospho-DL-homoserine was extracted subsequently with ethyl acetate for at least 6 hours, and then the solution of ethyl acetate was concentrated *in vacuo*. Crude DNP-*O*-phospho-DL-homoserine was purified by paper chromatography in phenol, subsequently in butanol-acetic acid as the solvents. To the eluate from the paper chromatogram were added 0.5 ml. of 2 *M* brium acetate and 1.5 volumes of alcohol. The barium salt of DNP-*O*-phospho-DL-homoserine was washed successively with 50 per cent alcohol, 95 per cent alcohol and finally with ether. The yield was about 30 mg. A free ester was obtained by dissolving the barium salt in diluted HCl and decomposing with calculated amount of sodium sulfate, followed by paper chromatography as described above. (Found: P, 8.1 per cent. Calculated for $C_{10}H_{12}O_{10}N_3P$: P, 8.5 per cent).

RESULTS AND DISCUSSION

Nature of An Intermediary Product

Enzymatic Synthesis and Isolation of Intermediate—As previously reported (*2*), it was observed that the incubation of homoserine and ATP with *Enzyme I* in the presence of Mg^{++} resulted in the formation of an intermediary precursor for threonine. When the deproteinized reaction mixture was subjected to paper chromatography, a new ninhydrine-positive spot was detected on paper chromatogram. Its Rf value was 0.14 in phenol and easily distinguished from that of homoserine, 0.58. On the basis of a preliminary test, this substance was found to correspond to the intermediary product which is converted to threonine by *Enzyme II*. Therefore, an attempt was made to isolate it from the reaction mixture to provide an information as to the nature of the postulated reactions.

In the case of large scale experiments, the Reaction (1, a) was carried out in a medium containing the following constituents per 160 ml; 4 mM of DL-homoserine, 1 mM of $MgSO_4$, 1 mM of NaF, 2.5 mM of potassium phosphate, pH 6.8, 1 mM of ATP and *Enzyme I* with 20 mg. of protein-nitrogen. The reaction was carried out at 37° for 3 hours and stopped by heating on a boiling water bath for 5 minutes. The precipitate was removed by centrifugation.

The intermediate was then separated as barium salt from the deproteinized supernatant by the following procedure: Inorganic phosphate was precipitated by adding 50 ml. of magnesia mixture at pH 9 and removed by centrifugation. To the supernatant were added 10 ml. of 2 *M* barium acetate and 3 volumes of alcohol and cooled overnight. The precipitate containing the barium salt of the intermediate was collected by centrifugation, washed twice with 60 per cent alcohol. The precipitate was then treated with approximately 30 ml. of 0.05 *N* HCl and insoluble material was centrifuged off. The supernatant was brought to pH 8.0 with 5 *N* NaOH and the barium salt of intermediate was again precipitated by the addition of 3 volumes of alcohol. After chilled for an hour, the suspension was centrifuged and the supernatant was discarded. This precipitate was dissolved in approximately 20 ml. of 0.05 *N* HCl and barium ion was removed off by the addition of 10 per cent sodium sulfate. The supernatant was then neutralized with *N* potassium carbonate and the intermediate was precipitated as mercury salt of carbamic acid derivative by the alternate addition of 20 per cent

mercury acetate and N potassium carbonate. This precipitate, which was contaminated by large amounts of yellow mercury carbonate, was washed repeatedly with water and 50 per cent alcohol, suspended in 30 ml. of water and decomposed with hydrogen sulfide. The mercuric sulfide is centrifuged, washed once with approximately 5 volumes of water, and discarded. The combined supernatant and washings were neutralized with N NaOH, treated with charcoal, and filtered. To the clear filtrate were added 2 ml. of 2 M barium acetate and 3 volumes of alcohol at pH 8.0. After cooled overnight in a refrigerator, the suspension was centrifuged and the precipitate was washed successively with 60 per cent alcohol, 95 per cent alcohol, and finally with acetone. The dried product weighed 160 mg. and was an almost white amorphous powder. Analytical results showed that the isolated sample was almost free of inorganic phosphate and adenine derivatives; inorganic phosphorus was less than 0.5 per cent, adenine was 0.01 per cent as determined spectrophotometrically at 260 mμ. These contaminates could be removed by paper electrophoresis at pH 7.0. The isolated material gave single ninhydrine-positive spot on one-dimensional chromatography in phenol and butanol-acetic acid and on paper electrophoresis at pH value ranging from 2.2 to 9.0.

Results of Acid Hydrolysis—In order to elucidate the nature of the intermediate, experiments were undertaken in which the isolated sample was subjected to acid hydrolysis under various conditions. About 500 μg. of the isolated sample was dissolved in 6 N HCl and heated in a sealed tube at 105° for 20 hours. The hydrolyzate was then dried *in vacuo*, and taken up in 0.5 ml. of water. Aliquots of 0.05 ml. were subjected to paper chromatography in phenol and butanol-acetic acid. A new ninhydrine-positive area was detected on paper chromatograms. This spot was identified as homoserine by co-chromatography with known substances. Since complete separation of homoserine from threonine was not always achieved, further characterization of homoserine was made by its conversion into α-amino-γ-butyrolactone (*7*). Its Rf value is 0.85 in phenol and give a characteristic yellow color with ninhydrine.

In consideration of the possibility that threonine or threonine-like substances might be liberated from the intermediate by acid hydrolysis, approximately 300 μg. of the isolated sample was subjected to periodate treatment (*3*) before and after acid hydrolysis, but no significant formation of acetaldehyde was observed in both cases. The test for in-

organic phosphate was almost negative when applied to the unhydrolyzed material, but, after hydrolysis, liberation of inorganic phosphate was always observed.

Similar studies were carried out after varying periods of heating with either 1 or 6 N HCl at 100° to 105°. Typical results of hydrolyses with 6 N HCl for the periods of 1,3,5,8,20 and 30 hours showed the gradual liberation of homoserine and inorganic phosphate (Table I). Complete hydrolysis was made by heating for more than 30 hours. Treatment with N HCl at 100° for 20 minutes, a condition under which most acid-labile phosphate was known to be split, did not cause any liberation of inorganic phosphate. The gradual liberation of homoserine and inorganic phosphate under the above conditions would rule out the existence of an acyl phosphate linkage in the molecule of the intermediate.

TABLE I

Progressive Acid Hydrolysis of the Intermediate

Time	Liberation of inorganic phosphate
hrs.	*per cent*
1	12.5
3	23.1
5	34.2
8	57.6
20	95.0
30	99.5

Approximately 500 μg. of the isolated intermediate was dissolved in 6 N HCl and heated in a sealed tube at 100° for 1 to 8 hours or at 105° for 20 to 30 hours.

To obtain further information, the following experiment was attempted. To an aliquot containing approximately 600 μg. of the isolated sample in a microbeaker were added a few crystals of sodium nitrite and 0.2 ml. of 6 N HCl. After stirring for 10 minutes the content was evaporated to dryness *in vacuo*. The residue was taken up in 6 N HCl, hydrolyzed in a sealed tube at 100° for 20 hours, and subjected to paper chromatography. No ninhydrine-reactive substance was found on the developed chromatograms. This experiment indicated that the amino group of homoserine is free in the oringal compound and ruled out the possibility of peptide-like linkage between two molecules of this amino acid or of a N-P bond between homoserine and phosphorus. The

latter possibility was also excluded by the fact that the intermediate was quantitatively converted to 2,4-dinitrophenyl (DNP) derivative according to the method of Mills (*6*), as will be noted in the later section. These findings, together with the exclusion of acyl-phosphate, seem to indicate that the intermediate is a compound in which hydroxyl group of homoserine is phosphorylated.

Analysis of Isolated Intermediate—The barium salt separated from the reaction mixture was further purified by precipitating as mercury salt and decomposing with hydrogen sulfide, followed by converting to barium salt, as described above. Analytical data on the composition of the product were as follows:

	C	H	N	$P_{org.}$	$P_{inorg.}$
Found	12.3	3.0	3.7	8.4	0.4
Calculated for $C_4H_{10}O_7PNBa$	13.6	2.9	4.0	8.8	0

These values indicate that the nitrogen and carbon contents are approximately consistent with that of homoserine monophosphate, if it is assumed that the molar ratios of homoserine and phosphorus are 1:1. The simplest structure which would fit the analytical data is that of barium salt of *O*-phosphohomoserine monohydrate.

Identification with Synthetic Substances—In order to confirm the postulated structure, the synthesis of *O*-phosphohomoserine was attempted by the phosphorylation of *N*-formyl-DL-homoserine, followed by the hydrolysis of the formyl residue under mild conditions. The phosphorylation was carried out in alkaline aqueous solution in the presence of MgO or CaO. More potent phosphorylating agents, such as diphenylchlorophosphonate and dibenzylchlorophosphonate, were not employed, considering that homoserine might be converted to α-amino-γ-butyrolactone under the anhydrous conditions where these agents are active (*8*). Unfortunately, although a variety of conditions were tried, the yield was very low, and the desired product could not be obtained in an analytically pure form. This crude product, however, did not contain free homoserine and formylhomoserine, and gave inorganic phosphate and homoserine as a sole ninhydrine-reactive substance by acid hydrolysis in the usual manner. Since phosphorylation was considered to occur only at hydroxyl group of homoserine under the experimental conditions used, it is quite certain that the product was *O*-phosphohomoserine. The synthetic substance as well as isolated intermediate proved to act as a substrate for *Enzyme II*, by which they were converted to

threonine with simultaneous liberation of inorganic phosphate.

Since synthetic *O*-phosphohomoserine was not so far obtained in a pure form, more detailed identification of the intermediate was carried out after it was converted to DNP derivative. The DNP derivative of the intermediate was prepared from the isolated sample and 2,4-dinitrofluorobenzene, according to the method of Mills (*6*) in nearly quantitative yield. The product was extracted with ethyl acetate from the acidified reaction mixture and purified by paper chromatography according to the procedure employed in the synthesis of DNP-*O*-phospho-DL-homoserine (see "Experimental"). The yellow colored area on the paper chromatogram was eluted with water and the eluate was, if necessary to be condensed, subjected to the following studies.

Ultraviolet absorption spectrum of the compound was nearly identical with those of DNP-homoserine and DNP-*O*-phosphohomoserine having maxima at approximately 265 mμ and 360 mμ, which are known to be characteristic to DNP derivatives of aliphatic amino acid. On the assumption that the molar extinction coefficient for DNP-intermediate at 360 mμ is identical with that for DNP-homoserine, 1.69×10^4 in 4 per cent sodium bicarbonate, analysis of DNP-intermediate for phosphorus, nitrogen and DNP-homoserine indicated that the molar ratio of these three constituents were 1 :2.91 :1.09. These values are consistent with the ratio 1 :3 :1 as calculated from the postulated structure.

All compounds of interest were then subjected to paper chromatography and paper electrophoresis. Their Rf values and mobilities were compared simultaneously on the same paper in each solvent and at each pH level. Typical results are summarized in Table II. Both synthetic *O*-phosphohomoserine and DNP-*O*-phosphohomoserine showed the strictly identical behaviors with the isolated intermediate and its DNP-derivative, respectively. In paper electrophoresis these four compounds showed a greater anodic migration than homoserine and DNP-homoserine. These facts are in accordance with the postulated structure of the intermediate, which contains monoester phosphate group.

Thus, from all evidences presented above, the intermediary product between homoserine and threonine was identified as *O*-phosphohomoserine.

Studies on Phosphate Transfer

Phosphate Transfer from ATP to Homoserine—From the above findings

TABLE II

Rf Values and Comparative Mobilities of the Intermediate and Related Compounds in Paper Chromatography and Paper Electrophoresis

Compounds	Rf values on paper chromatography			Comparative mobilities on paper electrophoresis			
	Phenol	Acetic acid-butanol	Butanol-ammonia	pH 2.2	pH 5.0	pH 6.6	pH 9.0
				mm.	*mm.*	*mm.*	*mm.*
Intermediate	0.14	0.13	—	+40	+73	+94	—
O-Phosphohomoserine	0.14	0.13	—	+40	+73	+94	—
Homoserine	0.58	0.29	—	−16	−13	−15	—
DNP-Intermediate	0.34	0.43	0.01	+65	+102	+97	+118
DNP-*O*-Phosphohomoserine	0.34	0.43	0.01	+65	+102	+97	+118
DNP-Homoserine	0.71	0.81	0.41	+35	+55	+41	+ 65

Electrophoresis was carried out at 350 to 450 volts and comparative mobilities were expressed as distance from origin towards anode (+) or cathode (−). No attempt was made to regulate current flow, which varied from 26 to 50 mA. Buffer solutions used at each pH level were as follows: pH 2.2, 0.5 *M* sodium acetate—HCl; pH 5.0, 0.2 *M* sodium acetate—acetic acid; pH 6.8, 0.1 *M* potassium phosphate; pH 9.0, 0.15 *M* sodium borate —HCl.

the primary step of the reaction leading to the synthesis of threonine from homoserine can be formulated as in Reaction (2, a,) and therefore *Enzyme I* may be designated as "homoserinekinase."

(2,a) $\mathrm{CH_2OH}$ | $\mathrm{CH_2}$ | $\mathrm{CHNH_2}$ | COOH + ATP $\xrightarrow[\text{Homoserine-kinase}]{\mathrm{Mg^{++}}}$ $\mathrm{CH_2OPO_3H_2}$ | $\mathrm{CH_2}$ | $\mathrm{CHNH_2}$ | COOH + (ADP)

Since the role of ATP required in the over-all reaction was thus elucidated by the participation of its heigh-energy phosphate (~P) in the phosphorylation of homoserine, it seemed desireble to provide the additional evidences for direct phosphate transfer from ATP to homoserine, resulting in the formation of *O*-phosphohomoserine. This was accomplished by the following experiments in which the disap-

pearance of homoserine was compared with that of ~P (Table III) and the disappearance of ~P and the appearance of *O*-phosphohomoserine were also determined (Table IV).

TABLE III

Disappearance of High Energy Phosphate in Relation of Homoserine Utilization

Time	$-\Delta$ 10′P*	$-\Delta$ Homoserine
min.	μM	μM
10	1.62	1.7
20	2.57	2.4
30	3.28	3.1
50	4.07	4.2
70	4.07	—

Reaction mixture contained, expressed as micromoles, 9 DL-homoserine, 5 $MgSO_4$, 5 NaF, 25 tris-(hydroxymethyl)-aminomethane and homoserinekinase with 370 μg. of protein nitrogen. After incubated at 37°, the reaction was stopped by adding 2.0 ml. of 10 per cent trichloracetic acid, and aliquots of the supernatant were subjected to the estimation of phosphate and homoserine.

* $-\Delta$ 10′P refers not to the net change of labile phosphate, but to the disappearance of the sum of ~P and inorganic phosphate which was measured after 10 minutes hydrolysis. This may represent the increase of organic stable-P.

Since *O*-phosphohomoserine proved to be resistant to hydrolytic destruction, the rate of ~P transfer to homoserine was expected to be followed by measuring the disappearance of acid-labile phosphate in the reaction systems. However, it was observed that inorganic phosphate was liberated during the incubation period both in the absence and presence of homoserine, though the extent was at most 10 per cent of ~P added, and that the hydrolysis in the absence of homoserine was always greater than that in the presence. This may be ascribed to contaminating ATP-ase, because the sum of ~P and inorganic phosphate were found to be kept constant during incubation period in the absence of homoserine. The addition of NaF could not completely depress the hydrolytic activity. On the other hand, *O*-phosphohomoserine synthesized in the presence of homoserine proved not to be split with homoserinekinase preparation. These observations may indicate that the extent of ~P transfer from ATP to homoserine can be followed by es-

TABLE IV

High Energy Phosphate Transfer in Relation to the Formation of Intermediate

Time	$-\Delta$ 10′P	Total counts of phosphohomoserine formed	Δ Phospho-homoserine
min.	μM	*c.p.m.*	μM
10	0.65	1.19×10^3	0.64
20	1.32	2.37×10^3	1.27
30	1.77	3.12×10^3	1.68
60	2.75	4.84×10^3	2.60
90	3.20	5.75×10^3	3.10

Reaction mixture contained, expressed as micromoles, 8 DL-homoserine, 3.1 P^{32}-labeled ATP having specific activity of 1.77×10^3 c.p.m. per μM of labile phosphate, 30 diethylbarbiturate (pH 7.1), 16 NaF, 8 $MgSO_4$, and homoserinekinase with 240 μg. of protein nitrogen. Other conditions were the same as noted in Table III.

* Calculated from the specific activity of P^{32}-labeled phosphohomoserine isolated from the reaction mixture, 1.86×10^3 c.p.m. per micromole.

timating the increase of organic stable phosphate.

As shown in Tables III and IV, stoichiometric correspondence between the disappearance of the reactants and the appearance of the resultant is in substantial agreement with the formulation of Reaction (2, a).

By the aid of radioactive phosphorus a direct evidence of phosphate transfer from ATP to homoserine has also been provided. Homoserine and P^{32}-labeled ATP were incubated with *Enzyme I*, and P^{32}-labeled *O*-phosphohomoserine formed was separated as barium salt from the deproteinized reaction mixture, purified by paper electrophoresis at pH 7.0. The specific activity of the isolated sample was 1.86×10^3 c.p.m. per micromole of phosphate and proved to be consistent with that of ATP^{32} initially used, 1.77×10^3 c.p.m. per micromole of labile phosphate. On the other hand, the radioactivity of *O*-phosphohomoserine, isolated from the reaction mixture incubated under the same condition except that non-labeled ATP and inorganic P^{32} -phosphate were added, was negligible within the experimental error. It must be, therefore, concluded that the phosphate group of *O*-phosphohomoserine is derived only from the labile phosphate of ATP and does not exchange with inorganic phosphate present in the medium.

Threonine Formation from O-Phosphohomoserine—The data are shown in Table V. The results indicate that the reaction does not require ATP and Mg^{++}, and that it is accompanied by the simultaneous liberation of inorganic phosphate. The reaction is therefore formulated as in Reaction (2, b).

TABLE V

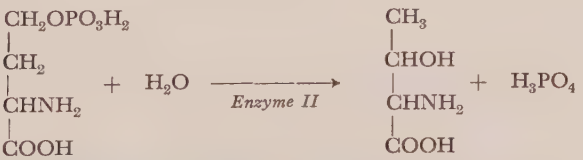
Formation of Threonine from O-Phosphohomoserine Accompanied by the Liberation of Inorganic Phosphate

Expt. No.	Time	Condition							
		Control		$+Mg^{++}$		+Versene		$+Mg^{++}$, +ATP	
		ΔPi*	ΔThreonine	ΔPi	ΔThreonine	ΔPi	ΔThreonine	ΔPi	ΔThreonine
	min.	μM	μM	μM	μM	μM	μM	μM	μM
1.	30	1.20	0.58	1.22	0.57	0.81	0.75	—	0.58
2.	30	2.43	1.70	2.31	1.52	2.04	1.87	—	—
	45	2.70	2.10	2.62	1.83	2.60	2.46	—	—
3.	15	2.04	0.97	2.16	0.75	1.55	1.06	—	—
	30	3.30	1.65	3.16	1.55	2.45	1.90	—	—
	45	4.25	2.10	4.11	2.10	—	—	—	—

Control system of Experiment 1 contained 1.5 μM of *O*-phosphohomoserine, 75 μM of diethylbarbiturate, pH 7.0, and lyophylized preparation of *Enzyme II*, which has been stored for 3 months in a desiccator, with 420 μg. of protein nitrogen in a final volume of 2.0 ml. Versene, $MgSO_4$ and ATP were added 10 μM, 20 μM and 5 μM, respectively. Reactions were performed at 37° and stopped by adding 2.0 ml. of trichloracetic acid. *Experiments* 2 and 3 were conducted under the same condition except that 4 μM of *O*-phosphohomoserine and freshly prepared *Enzyme II* with 360 and 410 μg. of protein nitrogen were added, respectively.

* The formation of inorganic phosphate.

(2,b)



As can be seen from the data given in *Experiments 1,2* and *3*, Table V, which were conducted with different enzyme preparations, the liber-

ation of inorganic phosphate was always found to exceed considerably the formation of threonine except for the results obtained in the presence of versene. A possible explanation is that it is probably due to the contaminating phosphomonoesterase which hydrolyzes the phosphate ester of the substrate added, and the addition of versene depresses the hydrolytic activity. An alternative explanation is that the splitting of phosphate occurs at the primary step of the threonine synthesis from *O*-phosphohomoserine, at which the homoserine moiety is simultaneously transformed into an active intermediate which is then converted to threonine. But this seems to be less likely because this cannot explain such a result that the addition of versene, on the contrary, increased the formation of threonine. Nevertheless, it is not still excluded that, as suggested by Teas and Horowitz (*9*), the transformation of homoserine into threonine may involve an initial dehydration to β-γ-unsaturated amino acid and a successive rehydration to threonine. The reaction appears to be novel in many respects and it is not desirable to discuss the mechanism until a possible role of phosphate in the -OH transfer is clarified. Further studies which settle this point are in progress.

SUMMARY

1. The intermediary product between homoserine and threonine, synthesized enzymatically from homoserine and ATP in the presence of Mg^{++}, was isolated as barium salt and identified as *O*-phosphohomoserine.

2. The over-all reaction leading to the synthesis of threonine has been formulated as homoserine$\longrightarrow$*O*-phosphohomoserine$\longrightarrow$threonine. The postulated pathway was also supported by the studies on phosphate transfer.

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STUDIES ON DENITRIFICATION

II. PRODUCTION OF NITRIC OXIDE AND ITS UTILIZATION IN THE N-N-LINKAGE FORMATION BY DENITRIFYING BACTERIA*

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Production and utilization of nitric oxide by denitrifying bacteria were recently reported by some authors (*1*, *2*, *3*, *4*). In our previous paper (*5*) we made some report on the nature and mode of action of denitrifying enzyme, which was investigated using leuco-toluylene blue as the hydrogen donor.

In the present work we found that the gas produced from nitrite in the presence of toluylene blue contains a large quantity of nitric oxide besides nitrogen and that toluylene blue inhibits the conversion of nitric oxide to nitrogen when lactate is given as the hydrogen donor. Furthermore, it was shown that, among various reducing agents tested, *p*-phenylene diamine and dimethyl-*p*-phenylene diamine besides leuco- toluylene blue can act specifically as hydrogen donors for the nitrogen production.

On the basis of these facts the probable mode of action of denitrifying enzyme, or exactly speaking, a system of enzymes will be discussed.

MATERIALS AND METHODS

The microorganism, preparation of its resting cell suspension and cell-free extract and the method of estimation are the same as described in the previous paper under the condition of strict exclusion of oxygen in the gas phase using Warburg apparatus (*5*). Most experiments were carried out with resting cells.

Nitric oxide was prepared from acidified ferrosulfate and sodium nitrite (*6*).

Gas analysis was carried out in the following procedures[1]. Amounts of CO_2

* This work was announced at the Annual Meeting of the Botanical Society of Japan held in Hiroshima on Oct. 13, 1955.

1) We owe the gas analysis to Assist. Prof. Dr. T. Koyama of the Chemical Institute, Faculty of Science, Nagoya University.

and O_2 were measured by the reduction of gas volume after the absorption in strong alkali and in alkaline pyrogallol respectively. The remaining gas was supposed to consist of CH_4, H_2, and N_2 after these procedures. By exploding with O_2 or air and H_2 of known volumes mixed, CH_4 was estimated as CO_2. Hydrogen content was obtained by calculation from the contraction of gas volume. It was assumed that the rest of gas consisted only of nitrogen.

RESULTS

Nitric Oxide Production—On measuring the gas production from nitrite and lactate in presence of toluylene blue, we observed a slow absorption of once evolved gas when the gas production nearly ended. So we attempted to examine whether or not nitric oxide was produced in this system and we could detect a large quantity of it, using absorbing agents of nitric oxide (Fig. 1).

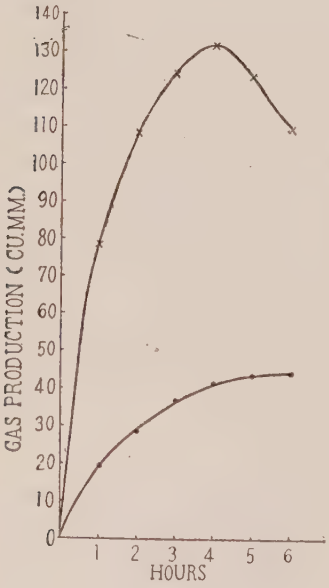


FIG. 1. Gas (nitrogen and nitric oxide) production in presence (—●—●—) and absence (—×—×—) of acidified $KMnO_4$ to absorb nitric oxide.

Phosphate *M*/2, pH 7.1, 0.3 ml.; sodium lactate *M*/2, 0.3 ml.; $NaNO_2$ *M*/22.4, 0.3 ml.; toluylene blue 10^{-2} *M*, 0.3 ml. in the main compartment.

Resting cell suspension 0.3 ml. in the side arm.

20 per cent KOH 0.2 ml. in the second side arm.

Saturated $KMnO_4$ solution acidified by H_2SO_4, 0.2 ml. in center well.

Total volume: 3.4 ml. or 3.2 ml.

Gas phase: nitrogen.

The reaction was started by the tipping of the cell suspension.

Of the agents used for nitric oxide absorption, acidified potassium permanganate and alkaline sodium sulfite solution gave approximately the same results. But acidified ferrosulfate was found to be unsuitable for nitric oxide absorption in our procedure.

In the present experimental condition all of the added nitrite was not always converted into gaseous product.[2)]

Utilization of Nitric Oxide and Its Inhibition by Toluylene Blue—In the gas phase of reaction vessels nitric oxide of various concentrations was applied in place of nitrite in the liquid phase, using lactate as the terminal reducer and without addition of any oxidation-reduction mediator. As shown in Fig. 2, in the concentration of 10 per cent (90 per cent nitrogen) the rate of nitric oxide absorption was at its maximum. The inefficient utilization of nitric oxide in higher concentration is not due to acid formation, for in the range of concentrations of nitric oxide less than 20 per cent, we could ascertain no appreciable shift of the pH in the liquid phase as revealed by examining after the experiments.

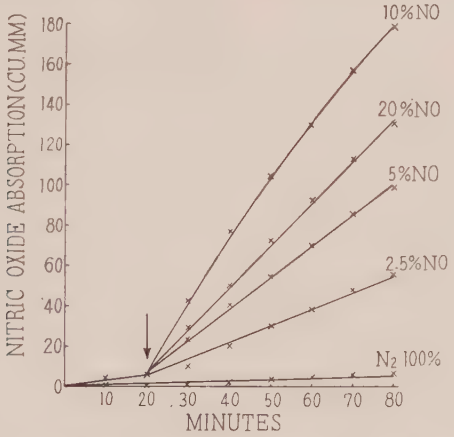


Fig. 2. Nitric oxide absorption at various concentration of nitric oxide in the gas phase.

Phosphate *M*/2, pH 7.1, 0.3 ml.; sodium lactate *M*/2, 0.3 ml. in the main compartment.

Resting cell suspension 0.3 ml. in the side arm.

20 per cent KOH in center well.

Total volume: 3.2 ml.

Proportions of nitric oxide to nitrogen in the gas phase are given on the right-hand side. At the arrow cell suspensions were added.

2) In the previous paper (*Ref. 5*, p. 376) the relation of the amount of gas evolved to that of nitrite added was somewhat erroneously stated. The apparent coincidence of the volume of gas as nitrogen with the latter is considered to be a matter of contingency.

The reaction of nitric oxide absorption was found to be intensely inhibited by toluylene blue (Fig. 3). That is, 10^{-3} *M* toluylene blue inhibits nitric oxide absorption up to about 70 per cent.

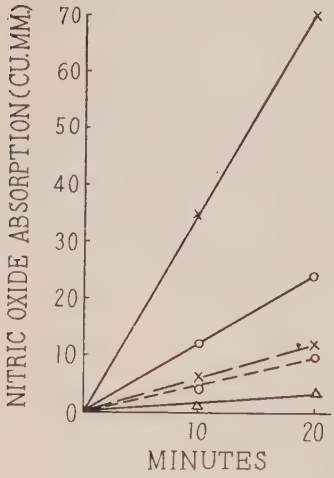


Fig. 3. Nitric oxide absorption in presence (—○—○—) and absence (—×—×—) of toluylene blue.

Phosphate *M*/2, pH 7.1, 0.3 ml.; sodium lactate *M*/2, 0.3 ml; toluylene blue 10^{-2} *M*, 0.3 ml. in the main compartment.

Cell suspension 0.3 ml. in the side arm.

20 per cent KOH 0.2 ml. in center well.

Total volume: 3.2 ml.

10 per cent nitric oxide in the gas phase.

Solid lines show the addition of sodium lactate.

In broken lines it was not added.

In the lowest line (—△—△—) cell suspension was not added.

These results have now brought to light why nitric oxide was liberated from nitrite in the presence of toluylene blue.

The rate of nitric oxide utilization was about twice as fast as that of nitrogen production from nitrite, if toluylene blue was not present.[3]

These findings make it highly probable that nitric oxide may be an intermediate in the process of denitrification and that the denitrifying reaction consists of at least two distinct steps, that is, the reduction of nitrous acid into nitric oxide and the conversion of the latter to nitrogen.

This conception was further corroborated by experimental results obtained with other hydrogen donors which have similar structures to that of leuco-toluylene blue, as elucidated in the following.

Phenylene Diamines as Hydrogen Donors—In the previous paper we could show a specific action of toluylene blue among several oxidation reduction dyes tested as an intermediary hydrogen carrier for the denitri-

3) The Michaelis constant for the denitrifying enzyme with nitrite as substrate at pH 7 and 30° was estimated to be about $10^{-3.6}$ mole/lit. As the concentration of nitrite used was *M*/224, the denitrifying enzyme must have been saturated with this substrate under these conditions.

fying reaction. We were interested in this structural specificity and investigated the applicability of other compounds. Hydroquinone, pyrogallol, ferrocyanide, aniline and *p*-aminophenol did not or could only slightly reduce nitrite. Among phenylene diamines only *p*-phenylene diamine and dimethyl-*p*-phenylene diamine could reduce nitrite specifically, while *o*- and *m*-compounds did not act at all, as shown in Fig. 4. The evolution of gas with either of these two hydrogen donors takes place at an enormously high rate. A reaction lag appeared in both cases, especially in the case of non-methylated *p*-phenylene diamine. The meaning of this lag is obscure at present.

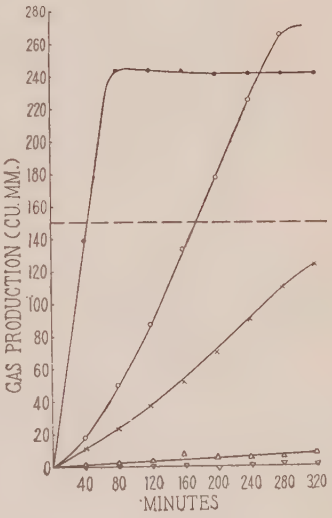


FIG. 4. Gas production using phenylene diamines as the reductants.

Phosphate *M*/2, pH 7.1, 0.3 ml.; sodium nitrite *M*/22.4, 0.3 ml.; hydrogen donor *M*/2, 0.3 ml. in the main compartment.

Cell suspension 0.3 ml. in the side arm. 20 per cent KOH in center well. Gas phase: nitrogen. Total volume: 3.2 ml.

—●—●— dimethyl-*p*-phenylene diamine.
—○—○— *p*-phenylene diamine.
—×—×— sodium lactate.
—△—△— *m*-phenylene diamine.
—▽—▽— *o*-phenylene diamine.

Broken line shows the quantity of the added nitrite-nitrogen. Phenylene diamines were used after neutralization of their hydrochlorides with NaOH.

By the manometric technique above mentioned nitric oxide was detected only in a small quantity (Fig. 5). For the purpose of analysis gas produced by these systems was gathered in fermentation tubes. The results of gas analysis showed the gas consisted of nitrogen for the most part and methane in a small quantity (Table I). Although the analytical procedure used was somewhat unsuitable for our purpose, the suspicion of the presence of nitrous and nitric oxide can be solved as in the following interpretation.

Nitric oxide which was detected in small quantity in the manometric technique, would have been consumed in long duration of experimental

TABLE I

Analysis of Gas Produced with the Nitrite-Phenylene Diamine System

Reductant	Dimethyl-*p*-phenylene diamine	*p*-Phenylene diamine
Sample taken	2.356 *ml.*	2.382 *ml.*
CO_2	0.00	0.00
O_2	0.00	0.00
H_2	0.00	0.00
CH_4	0.08 (3.4%)	0.04 (1.7%)
N_2	2.246 (96.6%)	3.378 (98.3%)

Reaction mixtures were prepared in fermentation tubes: Resting cell suspension 5 ml.; *M*/2 phosphate buffer at pH 7.1, 4.5 ml.; *M*/22.4, $NaNO_2$ 9 ml.; *M*/5, dimethyl-*p*-phenylene diamine or *p*-phenylene diamine hydrochloride 4.5 ml. (neutralized with NaOH before use.). Total volume: 45 ml.

The fermentation tubes were then incubated at 30° for 39 hours and brought into ice box before gas analysis was carried out after 24 hours.

courses in the fermentation tubes. Nitrous oxide should be suspected to be an intermediate in denitrification (*7*). If N_2O would have been produced, it would be detected in the fraction of CO_2 in the gas analytical data because of its large solubility in the strong alkali. As the content of gas revealed no CO_2 fraction at all, we were obliged to consider that there was no production of N_2O in our cases. Concerning more precise analysis on this problem we shall continue investigations.

On the basis of these considerations there can be no doubt to conclude that the greater part of gas produced consisted of nitrogen.

The quantity of gas produced in the presence of dimethyl-*p*-phenylene diamine and *p*-phenylene diamine corresponded to 1.6 and 1.8 times, respectively, of that of nitrite-nitrogen added.

As the most part of the gas is N_2, the extra-N should be derived from another source, namely from organic amine group of diamines in our cases. Then we come to the conclusion that the production of free nitrogen in the denitrifying system may have concern with a N-N-linkage formation between the phenylamino group and nitrite or nitric oxide.

Inhibition and Heat Destruction of the Enzyme in the Phenylene diamine-Nitrite System—Gas production by the dimethyl-*p*-phenylene diamine-nitrite system with cell-free extract was inhibited to 100 per cent either

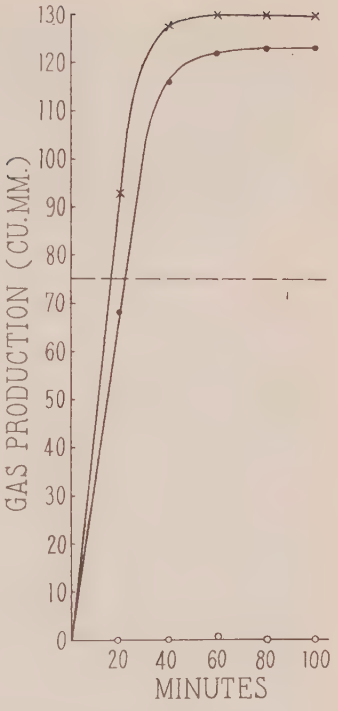


FIG. 5. Detection of nitric oxide and gas evolution with dimethyl-*p*-phenylene diamine in presence (●) and absence (×) of acidified $KMnO_4$.

Cell suspension 1 ml.; phosphate *M*/2, pH 7.1, 0.3 ml.; dimethyl-*p*-phenylene diamine hydrochloride *M*/2, 0.3 ml. (neutralized byNaOH), in the main compartment.

20 per cent KOH 0.2 ml. in the side arm.

$NaNO_2$ *M*/22.4, 0.15 ml. in the second side arm.

Acidified $KMNO_4$ 0.2 ml. in center well.

Total volume: 3.2 ml. or 3.4 ml.

Gas phase: nitrogen.

In the lowest line (—○—○—) cell suspension was heated at 80° for 10 minutes.

by 10^{-3} *M* KCN or by 10^{-3} *M* *p*-chloromercuribenzoate. Further the activity was eliminated completely by heating of the resting cell at 80° for 10 minutes (see Fig. 5). These properties of the enzymic reaction are almost the same as those of the ordinary lactate-nitrite system (see Table II).

Non-enzymatic Absorption of Nitric Oxide by p-Phenylene Diamines—Assuming the mechanism of N-N-linkage formation between the nitric oxide-N and phenylamino-N, we could expect no change of gas volume in the exchange reaction of nitric oxide with nitrogen. Although in the neutral range of pH any appreciable reaction cannot occur between nitrite and *p*-phenylene diamines in absence of the enzyme, nitric oxide in the gas phase readily reacts pure-chemically with *p*-phenylene diamines and is absorbed. In the case of *p*-phenylene diamine nitric oxide which was absorbed, was regenerated as some kind of gaseous products and this gas production seems to be markedly promoted by the bacteria, as

shown in Fig. 6a. But with dimethyl-*p*-phenylene diamine no such a re-evolution of gas could be detected (Fig. 6 b). The data fluctuated considerably but showed always similar tendencies when nitric oxide was used in concentrations of 10～2 per cent in the gas phase.

These phenylene diamines must be detroyed by some far-reaching reaction with such large quantity of nitric oxide as indicated by the absence of proper purple color which is always found to appear in the reaction mixtures of the phenylene diamines and nitrite with the bacteria.

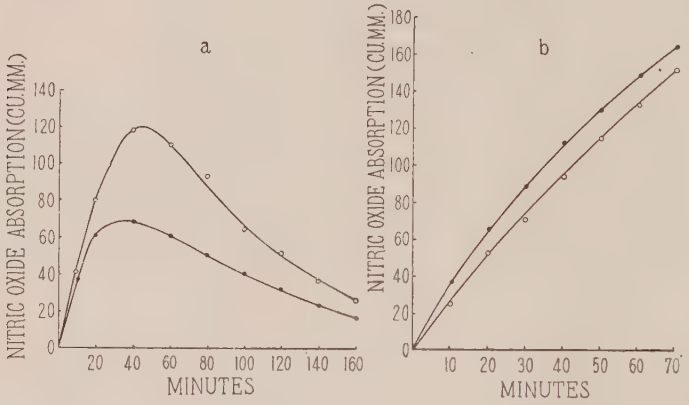


FIG. 6. Reactions between nitric oxide and *p*-phenylene diamines.
Phosphate *M*/2, pH 7.1, 0.3 ml; *p*-phenylene diamine *M*/2, 0.3 ml. (Fig. 6a) or dimethyl-*p*-phenylene diamine *M*/2, 0.3 ml. (Fig. 6b) in the main compartment.
Cell suspension 0.3 ml. in the side arm.
20 per cent KOH 0.2 ml. in center well.
10 per cent NO and 90 per cent N_2 in the gas phase.
Total volume: 3.2 ml.
—●—● Resting cell suspension was tipped in.
—○—○ It was not tipped in.

Effects of Various Inhibitors upon the Lactate-Nitrite System—Studies on the effects of various inhibitors on the reduction of nitrite, using lactate as hydrogen donor, revealed that both sulfhydryl group and some heavy metal should participate in the denitrifying reaction, as already suspected in the previous paper (*5*), (Table II). Namely KCN and diethyl-dithiocarbamate can exert strong inhibiting actions on the one hand, $CuSO_4$ and *p*-chloromercuribenzoate show also strong inhibition on the other.

TABLE II

Effects of Various Inhibitors

Inhibitors	Concentration	Inhibition
	M	%
KCN	10^{-3}	90
	10^{-4}	45
NaN_3	10^{-2}	20
NaF	10^{-2}	10
Sodium diethyl-dithiocarbamate	10^{-3}	90
Oxin	10^{-3}	0
CO (dark)	1 atom.	20
" (light)	1 atom.	No recovery
Monoiodoacetate	10^{-2}	50
p-Chloromercuribenzoate	10^{-3}	100
	10^{-4}	60
$CuSO_4$	10^{-4}	100

Activity was measured at 30° in the hydrogen or nitrogen atmosphere in the Warburg apparatus at pH 7 or 7.1. Fluoride inhibition was carried out at pH 6.8. Main cup: resting cell suspension 1 ml.; *M*/2, sodium lactate 0.3 ml.; *M*/4 or *M*/2, phosphate buffer at pH 7 or 7.1, 0.3 ml.; *M*/22.4, $NaNO_2$, 0.3 ml.

Side arm: inhibitor 0.3 ml.

Center well: 20% KOH 0.2 ml.

Total volume: 3.2 ml.

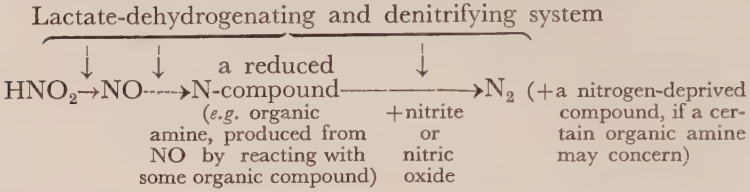
Measurements of the CO inhibition was started by tip-in of resting cell suspension.

DISCUSSION

From the results obtained *p*-phenylene diamines were found to be specific and efficient hydrogen donators in the denitrifying system of the bacteria, where the mode of function of these donators was shown to be of a particular nature in the sense that the production of molecular nitrogen was preceded by the formation of N-N-linkage between the phenylamino-N and nitrite-N or otherwise stating, nitrogen oxide-N.

If one may presume this kind of mechanism also in the lactate-nitrite

system, the fact that the amount of nitrogen produced by the lactate-nitrite system is equal to that of nitrite-N, indicates that a certain cyclic reaction including a formation of another nitrogen compound from nitric oxide with lactate must be concerned, as indicated in the following :



In the case of nitrite as the oxidant, nitric oxide produced will be utilized immediately when it is produced.[4)]

Furthermore, it must be worth while to be pointed out that the mode of action of the denitrifying system can be intimately related to that of cytochrome *c*-cytochrome oxidase system from the viewpoint of the same strict specificity for the *p*-phenylene diamine structure (*8*, *9*). Denitrifying bacteria, so far as known at present, are facultative anaerobes without exception and in fact it was shown that in the aerobic oxidations active oxygen uptake with our organism was observed only when dimethyl-*p*-phenylene diamine or *p*-phenylene diamine was used as a substrate, while *o*- and *m*-phenylene diamine could hardly be oxidized.

SUMMARY

1. Nitric oxide is produced from nitrite and is utilized in place of the latter and it is presumable that nitric oxide is an intermediate in denitrification.

2. Dimethyl-*p*-phenylene diamine and *p*-phenylene diamine can act specifically as reducing agents where the quantity of nitrogen produced corresponds to about twice as much as nitrite-N.

3. Gas production with the dimethyl-*p*-phenylene diamine-nitrite system is inhibited by KCN and *p*-chloromercuribenzoate. Effects of other various inhibitors were described.

4. The essential stages in the denitrifying process were discussed.

4) Component analysis of the enzyme system with dimethyl-*p*-phenylene diamine and nitrite as substrates has been also carried out by an ammonium sulfate fractionation of cell-free extract and the system was found to be divided into two protein components. The results will be reported before long.

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The Journal of Biochemistry, Vol. 43, No. 3, 1956

ON THE COMPONENTS OF BULL-FROG BILE (RANA CATESBIANA SHAW)

VI. CHEMICAL STRUCTURE OF α- AND β-TRIHYDROXY-HOMOCHOLENE

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(Received for publication, February 17, 1956)

Previously the author (*1*, *2*) isolated from bile of bullfrog three kinds of trihydroxyhomocholenes $C_{25}H_{42}O_3$. One of them melted at 177° and, upon catalytic hydrogenation, after absorbing 1 *M* of hydrogen it was converted to trihydroxyhomocholane of m.p. 185–6°. The latter was identified as 3 (α), 7 (α), 12 (α)-trihydroxyhomocholane, since the same substance could be obtained from cholic acid by Kolbe's electrolysis (*3*). Therefore this trihydroxyhomocholene was named α-trihydroxy-homocholene. On the other hand, at the catalytic hydrogenation of trihydroxyhomocholene of m.p. 238°, trihydroxyhomocholane of m.p. 199–201° was obtained after absorbing 1 *M* of hydrogen. It was oxidized with chromic acid, and triketohomocholane of m.p. 246–7° was obtained. The same triketohomocholane could be obtained from 3 (α), 7 (α), 12 (α)-trihydroxyhomocholane by chromic acid oxidation, and thus the triketohomocholene melting at 238° was named β-trihydroxyhomocholene.

The triketohomocholane was reduced by Huang-Minlon's method (*4*), and homocholane melting at 75° was obtained.

In this report the author describes on the position of the double bond and the steric configuration of three hydroxyl groups of α- and β-trihydroxyhomocholenes.

EXPERIMENTAL

Triketohomocholene—2 g. of α-trihydroxyhomocholene of m.p. 177° was dissolved in 50 ml. of glacial acetic acid, and 40 ml. of 5 per cent chromic acid solution in glacial acetic acid was added dropwise in 30 minutes. After 1 hour the excess chromic acid was reduced with hyposulfite, and the solution was put into a bulk of water. The precipi-

tate, after standing over night, was filtered, washed, dried, and crystallized from acetic acid. 1 g. of needle-shaped crystalls melting at 242° was obtained, which absorbed bromine promptly and reduced permanganate solution.

$C_{25}H_{36}O_3$	calcd.	C 78.08%, H 9.33%
	found	C 77.64%, H 8.95%

Two g. of β trihydroxyhomocholene was similarly treated, and 1.2 g. of needle-shaped crystals melting at 240–2° were obtained, which absorbed bronine promptly, reduced permanganate solution and showed no depression of the melting point on admixture with the above described ketoderivative from α-trihydroxyhomocholene.

$C_{25}H_{35}O_3$	calcd.	C 78.08%, H 9.33%
	found	C 77.54%, H 9.71%

Trihydroxyhomocholene Triacetate—0.5 g. of β-trihydroxyhomocholene was dissolved in 15 ml. of pyridine and 7.5 ml. of freshly distilled acetic anhydride were added. The solution was heated on a boiling water both for 24 hours and it was then put into a bulk of water. The precipitate was filtered, washed with water, dried, and crystallized from dilute methanol. 0.35 g. of flake-shaped crystals melting at 218° was obtained.

$C_{31}H_{48}O_6 1/2H_2O$	calcd.	C 70.82%, H. 9.39%
	found	C 70.87%, H 10.08%

3,7,12-Trihydroxy-14-keto-14 : 15-homocholanic Acid-15—0.3 g. of trihydroxyhomocholene triacetate of m.p. 218° was dissolved in 30 ml. of acetone and 10 per cent permanganate solution in acetone was added dropwise, until the color of permanganate no more discolored (about 20 ml. were necessary). The solution was then heated at 90° for 30 minutes, cooled and the excess permanganate was neutralized with hyposulfite. Water was added and the solution was concentrated to remove acetone. A small amount of precipitate (unchanged neutral substance) was filtered, and the filtrate was acidified with dilute hydrochloric acid. The precipitate was filtered, washed with water and dried. Trials to crystallize it being failed, it was hydrolyzed with 10 per cent alcoholic caustic soda. The solution was diluted with water and evaporated to remove alcohol. Dilute hydrochloric acid was added, and the precipitate was filtered, washed with water, dried and crystallized from dilute alcohol. One-tenth g. of needle-shaped crystals melting at 99–101° was obtained.

$C_{25}H_{42}O_6$ calcd. C 68.45% H 9.19%
found C 68.66% H 8.53%

3,7,12-Trihydroxy-14-keto-14 : 15-homocholanic Acid-15 *Monoxime*—An alcoholic solution of 50 mg. of 3,7,12-trihydroxy-14-keto-14 : 15-homocholanic acid-15 was heated on a water bath with 20 mg. of hydroxylamine hydrochloride and 30 mg. of sodium acetate for 3 hours. The solution was put into a bulk of water, and the precipitate was filtered, washed with water, dried and crystallized from dilute alcohol. 20 mg. of needle-shaped crystals decomposing at 133° were obtained.

$C_{25}H_{43}O_6N$ calcd. N 3.08 %
found N 3.19%

Trihydroxyhomocholene 3,7-Diacetate—200 mg. of α-trihydroxyhomocholene were dissolved in 10 ml. of glacial acetic acid, and 2 ml. of acetyl chloride were added. The mixture was shaken at room temperature for 2 days, and was put into a bulk of ice water. The precipitate was filtered, washed with water, dried and crystallized from dilute alcohol. 120 mg. of needle-shaped crystals of m.p. 180° were obtained.

$C_{29}H_{46}O_5$ calcd. C 73.37%, H 9.76%
found C 72.92%, H 9.89%

Similarly from 200 mg. of β-trihydroxyhomocholene, 100 mg. of plateshaped crystals melting at 94° were obtained.

$C_{29}H_{46}O_5$ calcd. C 73.37%, H 9.76%
found C 73.67%, H 10.02%

3,7-Dihydroxy-12-ketohomocholene—100 mg. of trihydroxyhomocholene 3,7-diacetate melting at 94° were dissolved in 3 ml. of glacial acetic acid, and 1 ml. of 5 per cent chromic acid solution in glacial acetic acid was added drop by drop during 30 minutes. After the solution was stood for 1 hour it was neutralized with hyposulfite and was put into a bulk of water. After standing over night, the precipitate was filtered, washed with water and dried. As the trial to crystallize it was failed, it was hydrolyzed with 10 per cent alcoholic caustic soda for 3 hours, diluted with water and concentrated to remove alcohol. Precipitate was formed, which was filtered, washed with water, dried and crystallized from dilute alcohol. 20 mg. of needle-shaped crystals of m.p. 197–8° were obtained.

$C_{25}H_{40}O_8 \cdot H_2O$ calcd. C 74.46%, H 10.62%
found C 73.84%, H 10.41%

Trihydroxyhomocholene 3,7-diacetate of m.p. 180° was similarly treated, but no crystalline product was obtained.

3 (α), 12 (α),-Dihydroxy-7-ketohomocholane——50 mg. of 3 (α), 7 (α), 12 (α)-trihydroxyhomocholane, which had been prepared from α-trihydroxyhomocholene and melted at 185–6° were dissolved in 5 ml. of alcohol and put into a three necked flask. A small amount of caustic soda solution and of bicarbonate solution was added, and 0.1 ml. of bromine solution, which had been prepared by dissolving 2 g. of bromine in 1 ml. of chloroform, was added drop by drop during 2 hours at-5-0° with stirring. The mixture was stirred for another 2 hours, then kept for 3 hours at room temperature, and neutralized with dilute hydrochloric acid. After being concentrated under reduced pressure, it was put into a bulk of water. The precipitate was filtered, washed with water, dried and crystallized from dilute alcohol. 20 mg. of needle-shaped crystals of m.p. 172° were obtained.

$C_{25}H_{42}O_3$	calcd.	C 76.87%, H 10.83%
	found	C 76.64%, H 10.96%

Trihydroxyhomocholane of m.p. 199–201°, which had been obtained from β-trihydroxyhomocholene by catalytic hydrogenation, was similarly treated.

Needle-shaped crystals of m.p. 172° were obtained, which showed no depression of the melting point when mixed with the sample obtained from trihydroxyhomocholane of m.p. 186°.

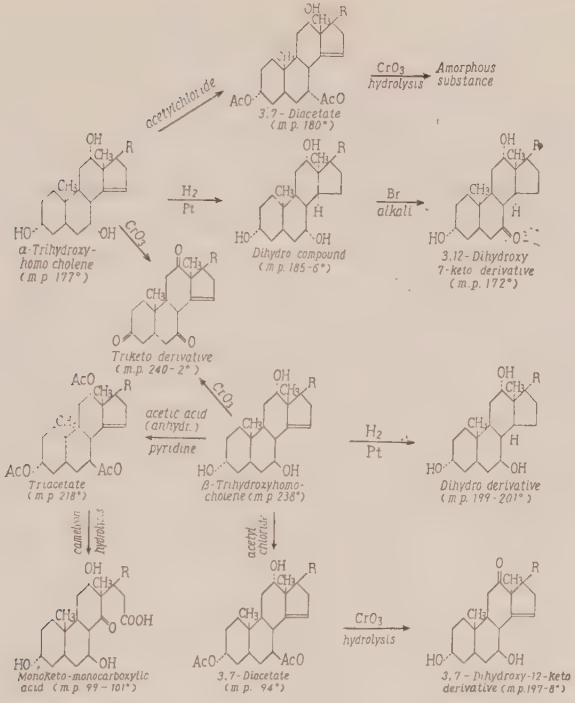
$C_{25}H_{42}O_3$	calcd.	C 76.87%, H 10.83%
	found	C 76.95%, H 10.76%

3 (α), 12 (α)-Dihydroxy-7-ketohomocholane Monoxime—An alcoholic solution of 20 mg. of 3 (α), 12 (α)-Dihydroxy-7-ketohomocholane was heated on a water bath with 8 mg. of hydroxylamine hydrochloride and 12 mg. of sodium acetate for 3 hours, and then put into a bulk of water, dried and crystallized from dilute alcohol. 10 mg. of needle-shaped crystals decomposing at 195° were obtained.

$C_{25}H_{43}O_3N$	calcd.	N 3.45%
	found	N 3.23%

RESULTS

The results obtained in the present experiment can be summarized as follows.



$$[R=-\overset{CH_3}{\overset{|}{C}}H-CH_2-CH_2-CH_2\text{-}COOH,\ A=CH_3O-]$$

DISCUSSION

Two trihydroxyhomocholenes, which had been obtained by hydrolyzing two different sulfates of m.p. 188° and 199° and named α- and β-respectively, yielded upon oxidation with chromic acid the same unsaturated ketone of m.p. 240–2°. Previously the author have reported, that either of α- and β-trihydroxyhomocholenes have one double bond at the same position and their C_3-hydroxyl groups are probably in α-configuration. Hence it can be said that α- and β-trihydroxyhomocholenes differ each other only in the steric configurations of the C_7- and C_{12}-hydroxyl groups.

Regarding the position of the double bond of β-trihydroxyhomocholene from the author's report V and from that of bufotalin (*5*), it may be either at C_8 or at C_{14}. While the tertiary hydroxyl group is most

likely to be at C_{14}, the double bond between C_8 and C_{14} is known to be resistant against hydrogenation. So it must be at C_{14-15} or C_{15-16} by migration.

The double bond of C_{14-15} should after oxidation give a monoketo monocarboxylic acid and that of C_{15-16} should give a dicarboxylic acid. β-Trihydroxyhomocholene was acetylated with pyridine and acetic anhydride to yield triacetate, which was oxidized and then hydrolyzed. 3, 7, 12-Trihydroxy-14-keto-14:15-homocholanic acid, which gave monoxime decomposing at 133°, was thus obtained. So the position of the double bond of β-trihydroxyhomocholene as well as its α-homologue must be at C_{14-15}. C_{14-15}-Double bond should yield by catalytic hydrogenation two cis-trans isomers concerning C_{14}-hydrogen against C_{13}-methyl group. By catalytic hydrogenation of dihydroxycholenic acid, R. K. Callow (*6*) obtained only desoxycholic acid alone, and the author (*1*, *2*) obtained trihydroxyhomocholane of m.p. 199–201° in 80% yield and homocholane melting at 75° from the latter. Therefore C_{14}-hydrogen of both trihydroxyhomocholane may probably have the same trans-position to C_{13}-methyl group as the usual bile acids.

To know the steric configuration of C_3 and C_7-hydroxyl groups of α- and β-trihydroxyhomocholenes by making their 12-ketoderivatives, they were acetylated with acetylchloride, and 3,7-diacetates melting at 180° and 94° respectively were obtained, which were oxidized with chromic acid and successively hydrolyzed. From β-trihydroxyhomocholene diacetate 12-ketoderivative was obtained in crystalline form, on the other hand from α-isomer no crystalline derivative was obtained.

3 (α), 7 (α), 12 (α)-Trihydroxyhomocholane and 3 (α), 7 (?), 12 (?)-trihydroxyhomocholane, which had been obtained by catalytic hyhydrogenation of α- and β-trihydroxyhomocholenes respectively, were oxidized with bromine at —5–0° according to W. M. Hocker's method (*7*). From either the same 7-ketoderivative was obtained.

Accordingly the starting two trihydroxyhomocholenes are steric isomers concerning their C_7-hydroxyl group. Therefore α-trihydroxyhomocholene may plausibly be 3 (α), 7 (α), 12 (α)-trihydroxyhomocholene and β-isomer be 3 (α),7 (β),12 (α)-trihydroxyhomocholene, and either of them has a double bond at C_{14-15}.

SUMMARY

1. From α- and β-trihydroxyhomocholenes, same keto-derivative was obtained, which means that either of trihydroxyhomocholenes has

a double bond at the same position. From β-trihydroxyhomocholene, after permanganate oxidation, 3,7,12-trihydroxy-14-keto-14:15-homocholanic acid-15 was obtained, which means, as indicated in the previous reports (*1*), (*2*), that the double bond is between C_{14} and C_{15}.

2. To know the steric configuration of 3-hydroxyl group of α- and β-trihydroxyhomocholenes, they were hydrogenated to yield 3 (α), 7 (α), 12 (α)-trihydroxyhomocholane and 3 (α), 7 (?), 12 (?)-trihydroxyhomocholane respectively, which upon oxidation with bromine gave same 7-ketoderivative of m.p. 172°. Therefore three hydroxyl groups of α-trihydroxyhomocholene are all in α-configuration and, among these three hydroxyl groups of β-isomer, that of C_7 has β-configuration.

α-Trihydroxyhomocholene is 3 (α), 7 (α), 12 (α)-trihydroxyhomocholene and β-trihydroxyhomocholene is 3 (α), 7 (β), 12 (α)-trihydroxyhomocholene.

The author is greatly indebted to President Dr. Toda for his encouragement and guidance, and to Dr. Shimizu, the President of Okayama University, for his permittance to use his laboratory at case. The author also thanks for the economical assistance of the Supporter's Association of our College.

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ON THE KINETICS OF THE HUMAN BLOOD CHOLINESTERASE

V. THE INHIBITION OF ACETYLCHOLINESTERASE AND CHOLINESTERASE BY HYDROGEN ION AND TETRAETHYLAMMONIUM BROMIDE

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(Received for publication, February 20, 1956)

The human blood cholinesterase has been classified into an erythrocyte specific cholinesterase (acetylcholinesterase) and serum nonspecific cholinesterase (cholinesterase). But the chemical or physiological differences of these two types of cholinesterase are so far not yet clear.

Through their studies on the acetylcholinesterase, Wilson, Bergmann and Nachmansohn (*1-5*) have suggested its active surface to be consisting in " anionic " and " esteratic " sites.

With respect to the difference in the chemical nature of the active surface of these two enzymes, the understanding is diversing among the investigators. Adams and Whittaker (*6*) concluded from a study of the specificity of both enzymes that there exists in acetylcholinesterase a negatively charged atom, which is absent from the active centers of the cholinesterase. Wilson (*7*), however, have considered that the picture of the enzyme-substrate complex which emerges for the cholinesterase is quite similar to that of acetylcholinesterase, but differs quantitatively in two important respects: the Coulomb forces of attraction and the size of the anionic site. From the recent studies of inhibition by methonium compounds, Bergmann and Segal (*8*) have shown that both enzymes contain an esteratic site of similar properties, but differ in the number of their anionic groups; the acetylcholinesterase containing two negatively charged groups, while the cholinesterase only one. We have presumed, however, that the active surface of acetylcholinesterase is similar to that of cholinesterase based on the results obtained by the present auther, previously (*9*, *10*).

In this communication, the results obtained from the experiments dealing with the inhibition of these two enzymes by hydrogen ion and tetraethylammonium bromide are described. From these experiments,

it was expected that kinetic study of the reaction between the enzyme and the hydrogen ion or tetraethylammonium bromide would not only clarify the differences of the two types of cholinesterase, but also contribute to a more precise definition of the active surface of these enzymes.

EXPERIMENTAL

Substrate: Commercial acetylcholine chloride was used.

Inhibitor: Commercial tetraethylammonium bromide was used. For the determination of tetraethylammonium bromide concentration, N content was estimated by the microkjeldahl method every time at use.

Enzyme preparations: Human erythrocyte was used as the enzyme source of acetylcholinesterase, partially purified according to the method of Mounter and Whittaker (*11*). As the cholinesterase, human serum was used.

Measurement of enzyme activity: For the determination of enzyme activity the colorimetric method described by Hestrin (*12*) was applied. Optical absorption was measured at 500 mμ by spectrophotometer of Hitachi Company. The temperature was held at 37° except in the experiment of temperature dependence.

Buffer: 0.1 *M* Phosphate and acetate buffer were used. The difference in the type of buffers gave no effect on the enzyme activity.

RESULTS

The Effect of the Hydrogen Ion Concentration upon the Enzyme Activity— Dependence of the activity of both acetylcholinesterase and cholinesterase on pH was measured with given substrate concentration. Fig. 1 shows a plot of enzyme activity thus measured against time. The activity of enzyme decreased gradually with increasing hydrogen ion concentration and the reaction proceeded lineally with time in each pH value of the media. Fig. 2 represents pH-activity curve of acetylcholinesterase and cholinesterase.

The pH-activity curve for the system of acetylcholinesterase and of cholinesterase has been so far reported by several investigators (*2*, *13–17*). From the present experiment by use of the colorimetric method, the optimum pH for both enzymes was found to be at 7.5–8.0. The shapes of these curves are both bell shaped. Considering the activity decreasing at acid side, the degree of inhibition, H, by hydrogen ion can be defined as follows:

$$H = 1 - v_H/v_o \qquad \text{Eq. (1)}$$

where v_o and v_H are velocity in the optimum pH and in the acid side, respectively.

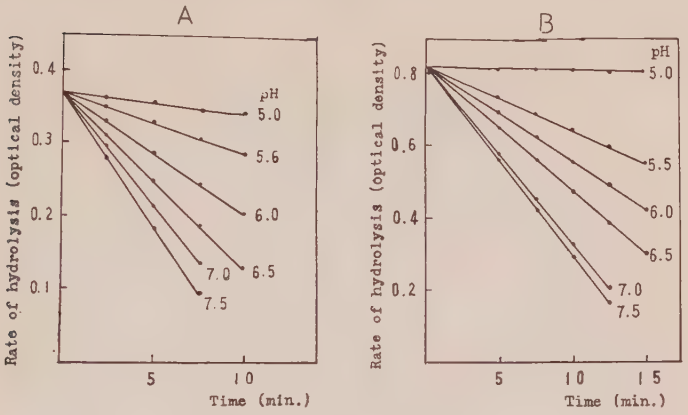

Fig. 1. The relationship between the activity and time at various pH values (A: acetylcholinesterase, [s]$=3.7\times10^{-3}$ *M*, B: cholinesterase, 37°, [s]$=8.2\times10^{-3}$ *M*).

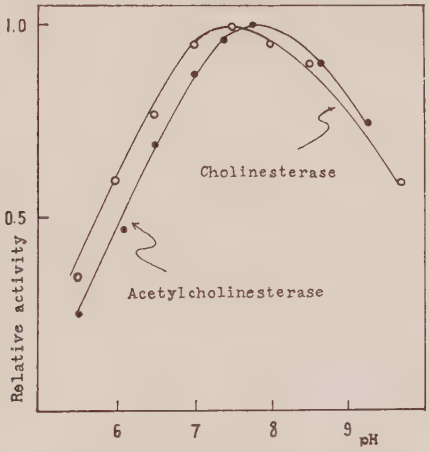

Fig. 2. pH-activity relation. Experiments carried out at substrate concentration of 5×10^{-3} *M* for acetylcholinesterase and 2.5×10^{-2} *M* for cholinesterase.

As may be seen in Fig. 3, the relationship between the degree of inhibition and the hydrogen ion concentration can be represented by a sigmoid curve of first order.

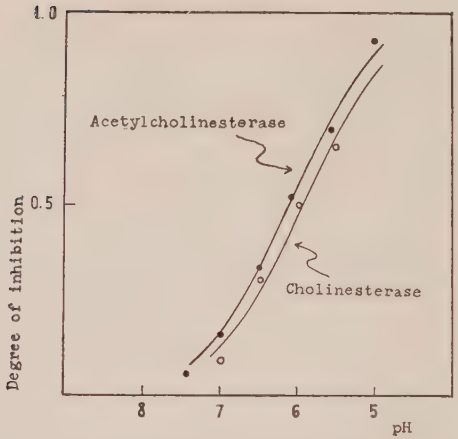

FIG. 3. The relationship between the degree of inhibition by hydrogen ion and pH. The initial substrate concentrations were 5×10^{-3} *M* for acetylcholinesterase and 1.5×10^{-2} *M* for cholinesterase.

Therefore, the degree of inhibition can now be represented by the following equation:

$$H=\frac{[H^+]}{\phi_H+[H^+]} \qquad \text{Eq. (2)}$$

where ϕ_H is a constant corresponding to hydrogen ion concentration at 50 per cent inhibition. Under the condition of the reaction given in Fig. 3, $p\phi_H$ of acetylcholinesterase and cholinesterase are 6.05 and 5.85, respectively. The relationship between the values of $p\phi_H$ and the concentration of substrate is shown in Table I.

$p\phi_H$ changed only little with the increase of substrate concentration. Therefore, the inhibition of both enzymes by hydrogen ion seems not to be competitive with substrate.

Now, taking $p\phi_H$ values as apparent dissociation constant, the effect of temperature upon these $p\phi_H$ values are shown in Fig. 4.

From this results, the heat of the dissociation, ΔH, are calculated as 8.5 kcal. for acetylcholinesterase and 6.5 kcal. for cholinesterase.

Inhibition by Tetraethylammonium Bromide—From the experimental results so far obtained, it may be assumed that the reactions of both enzymes with hydrogen ion are similar.

The relationship between the enzyme activity and time at various concentration of tetraethylammonium bromide is shown in Fig. 5.

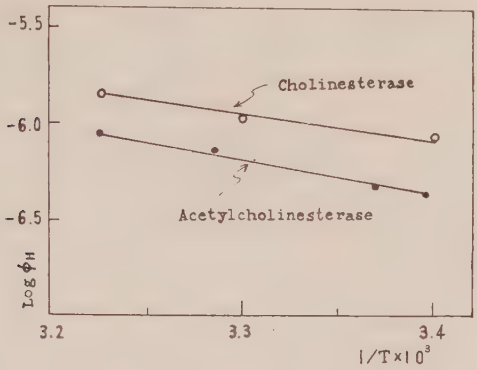

FIG. 4. The effect of temperature upon the ϕ_H values.

TABLE I

The Relationship between the Values of $p\phi_H$ and the Concentrations of Substrate

Acetylcholinesterase			Cholinesterase		
Substrate concentration	$p\phi_H$	pK_H*	Substrate concentration	$p\phi_H$	dK_H*
M			M		
8.0×10^{-3}	5.97	7.64	2.5×10^{-2}	5.75	7.09
7.6 „	6.05		1.5 „	5.85	6.98
5.0 „	6.05	7.35	0.8 „	5.90	6.78
3.7 „	6.07				
2.5 „	6.07	7.06			

* pK_H was calculated from the Eq.(13).

The enzyme activity decreased with increasing tetraethylammonium bromide concentration, the reactions being represented by straight line. The degree of inhibition may be defined as follows:

$$H = 1 - v_I/v_o \qquad \text{Eq. (3)}$$

where v_I and v_o are the velocity in the presence and absence of inhibitor. The relationship between H and inhibitor concentration could be represented with sigmoid curve of first order as shown in Fig. 6. That is, the degree of inhibition can be expressed by the following equation:

$$H = \frac{[I]}{\phi_I + [I]} \qquad \text{Eq. (4)}$$

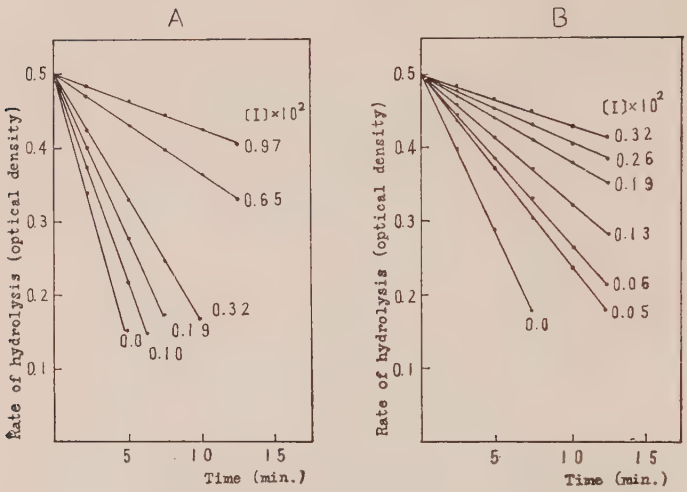

FIG. 5. The relationship between the activity and time at various concentration of tetraethylammonium bromide. The initial substrate concentration were 5×10^{-3} *M* for both enzymes.
(A: acetylcholinesterase, pH=7.5, 37°, B: cholinesterase, pH=8.0, 37°).

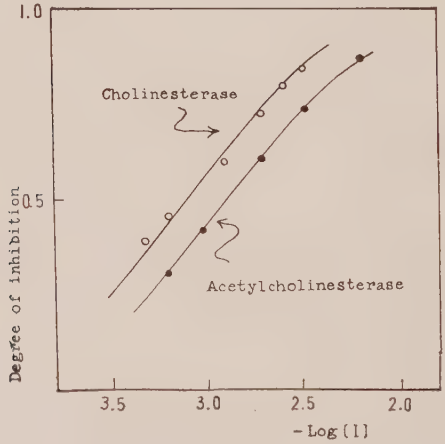

FIG. 6. The relationship between the degree of inhibition by tetraethylammonium bromide and its concentration. The initial substrate concentration were 3×10^{-3} *M* for acetylcholinesterase and 5×10^{-3} *M* for cholinesterase.

where [I] is molar concentration of tetraethylammonium bromide, ϕ_I is a constant which corresponds to the inhibitor concentration at the 50 per cent inhibition.

TABLE II

The Relationship between the Values of $p\phi_I$ and pK_I, and the Substrate Concentration

Acetylcholinesterase			Cholinesterase		
Substrate concentration	$p\phi_I$	pK_I	Substrate concentration	$p\phi_I$	pK_I
M			M		
7.5×10^{-3}	2.37	3.90	1.0×10^{-2}	2.90	3.87
5.0 "	2.59	3.89	7.5×10^{-3}	3.06	3.92
3.0 "	2.91	3.95	5.0 "	3.14	3.89

Herewith, the values of $p\phi_I$ under each substrate concentration were found as shown in Table II. As seen from Table II, the values of $p\phi_I$ decreased with increasing substrate concentration. Now, if the inhibition reaction by tetraethylammonium bromide proceeds in a similar way to that by eserine (*9*), the reaction may be expressed by the following equations:

$$E + S \rightleftarrows ES \qquad \text{Eq. (5)}$$

$$ES \xrightarrow{k_3} E + P \qquad \text{Eq. (6)}$$

$$ES + S \underset{K'_m}{\rightleftarrows} ESS \qquad \text{Eq. (7)}$$

$$E + I \underset{K_I}{\rightleftarrows} EI \qquad \text{Eq. (8)}$$

where K'_m and K_I are the dissociation constants of these equilibria in steady state, the velocity of the over all reaction is represented as follows:

$$v_I = k_3\,[\Sigma E]\,\left(1+\frac{K_m}{[S]}+\frac{[S]}{K_m'}+\frac{[I]K_m}{[S]K_I}\right)^{-1} \qquad \text{Eq. (9)}$$

where k_3, ΣE, and K_m are the velocity constant, the total concentration of the enzyme and the Michaelis constant, respectively.

Therefore, from Eq. (3), the degree of the inhibition is represented as follows:

$$H=\frac{[I]}{K_I\left(1+\frac{[S]}{K_m}+\frac{[S]^2}{K_mK_m}\right)+[I]} \qquad \text{Eq. (10)}$$

Comparison of this equation with Eq. (4) leads to the following relationship:

$$\phi_I = K_I \left(1 + \frac{[S]}{K_m} + \frac{[S]^2}{K_m \cdot K_m'}\right) \qquad \text{Eq. (11)}$$

As may be understood from this equations, $p\phi_I$ is represented as a function of the substrate concentration. As may be seen in the third and sixth columm of Table II, the values of pK_I calculated in varied substrate concentrations were obtained as constant and at the same order for both enzymes.

Therefore, it was indicated that the inhibition of the two enzymes by tetraethylammonium bromide is represented by Eq. (8), as in the case by eserine and that the pK_I is pH independent (Table III).

Fig. 7 is the plots of the pK_I against the reciprocals of absolute temperature. From this figure, the dissociation energies of inhibitor (ΔH) are calculated as 6.5 kcal. for acetylcholinesterase and 6.0 kcal. for cholinesterase.

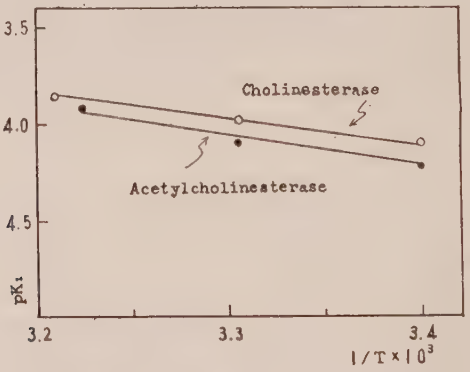

FIG. 7. The effect of temperature upon the pK_I values.

DISCUSSION

The effect of pH upon the acetylcholinesterase and cholinesterase has been discussed by many investigators, but their results have not always been in accord with each other.

pH-activity curves in Fig. 2 are different from that which was obtained from the experiments with purified acetylcholinesterase of electric organ of Electrophorus electricus by Wilson *et al.* (2); optimum pH

TABLE III

The Relationship between the Values of $p\phi_I$ and pK_I, and the pH

Acetylcholinesterase			Cholinesterase		
pH	$p\phi_I$	pK_I	pH	$p\phi_I$*	pK_I*
8.0	2.54	3.84	8.0	3.06	3.85
7.5	2.59	3.89	7.0	3.05	3.84
6.0	2.54	3.84	6.5	3.02	3.81

* In this calculation, Eq.(7) was neglected. As the K_m values of acetylcholinesterase and cholinesterase, 3.7×10^{-4} mole/lit. and 1.2×10^{-3} mole/lit. previously reported were used. K_m' value of acetylcholinesterase was 1.2×10^{-2} mole/lit. (*9*).

in the latter was found to be at 8–9. The results in Fig. 2 are in agreement with those of Heilbronn (*17*) and Bergmann (*15*). It has been assumed by Wilson (*5*) that the inhibition of acetylcholinesterase by hydrogen ion may be explained by the combination of the latter to the esteratic site of the enzyme molecule.

If so, the inhibition by hydrogen ion should be represented by a competitive process with the substrate. The reaction may then be represented by the following equations:

$$E + S \rightleftarrows ES \qquad \text{Eq. (5)}$$

$$ES \longrightarrow E + P \qquad \text{Eq. (6)}$$

$$ES + S \rightleftarrows ESS \qquad \text{Eq. (7)}$$

$$E + H^+ \rightleftarrows EH^+ \qquad \text{Eq. (12)}$$

A steady state solution of these kinetic equations leads to the following equation in a similar manner as in the case with Eq. (11).

$$\phi_H = K_H \left(1+\frac{[S]}{K_m}+\frac{[S]^2}{K_m K_m'}\right) \qquad \text{Eq. (13)}$$

As indicated by this equation, $p\phi_H$ is represented as a function of the substrate concentration, but pK_H values calculated from this equation were not at constant as shown in the third and sixth column of Table I. Therefore, it may be presumed that the inhibition of both enzymes by hydrogen ion takes place by a combination of hydrogen ion to a certain group of enzyme surface which is inert to the substrate, and that by this combination the formation of enzyme-substrate complex or its dissociation is inhibited. The heat of ionization (ΔH) of these

two types of cholinesterase are approximately the same value as shown in Fig. 4. These results allow us to infer that the certain chemical groups in the enzyme molecule reacting with hydrogen ion may be of the same nature.

The inhibition by tetraethylammonium bromide were reported by Bergmann *et al.* (*3*, *8*), and Hase (*18*). This inhibition was investigated repeatedly to differentiate the nature of the so called anionic site of these two types of cholinesterase in anticipation of a more precise definition of the active surface of the enzymes.

The affinity of the inhibitor to the enzymes was represented by Bergmann (*8*) with the inhibitor concentration at 50 per cent inhibition. As above mentioned, however, the inhibition of these enzymes by tetraethylammonium bromide is competitive with substrate, so that the inhibitor concentration effecting 50 per cent inhibition may be changed with varying concentrations of the substrate. A more presice comparison of the affinity of the inhibitor to both enzymes may be achieved by the calculation of their dissociation constants.

As seen in Fig. (6), the inhibition by tetraethylammonium bromide may be caused by the combination of one molecule of the inhibitor to one molecule of the enzyme. The reaction between these groups of the enzymes and the inhibitor are independent of the hydrogen ion concentration ; approximately the same values of pK_I for two enzymes have been obtained, these values being in accord with the data by Hase (*18*). Thus it may be assumed that the chemical groups of the two enzymes which interact with the cationic group of the substrate or of the inhibitor may be of the same nature.

Recently it was assumed by Bergmann (*8*) that cholinesterase contains a single, and acetylcholinesterase two anionic sites. From the results above mentioned, however, it may be more plausible that these two types of cholinesterase both contain a single anionic site.

The difference of the Michaelis constants of these two enzymes can not be explained by the present studies. But it may well be presumed that this difference of Michaelis constants are due to the difference of the structure of configuration of the protein moiety of the enzyme which is connected with the function of the active surface of the enzyme molecule.

On the activity decreasing at alkaline side, a further investigation is now in progress.

The authors wish to express their sincere gratitude to Prof. K. Kaziro for his

continual encouragement and advice during the course of this work. Our thanks are also due to Dr. Nakajima and Mr. Sato of Nippon Seiyaku Company, for the material supplied.

SUMMARY

1. Inhibition of the over all reaction of two enzymes, acetylcholinesterase and cholinesterase, by hydrogen ion and tetraethylammonium bromide was investigated from the kinetic point of view.

2. It is assumed that the inhibition at acid side is caused by hydrogen ion following the first order kinetics with response to hydrogen ion concentration.

3. This inhibition by hydrogen ion seems to be non competitive inhibition with substrate.

4. The inhibition by tetraethylammonium bromide is caused by the combination of one inhibitor molecule to one enzyme molecule.

5. pK_I of the reaction of tetraethylammonium bromide with acetylcholinesterase and cholinesterase was obtained at 3.91 and 3.88, respectively.

6. Based on the data obtained, the difference of the nature of the two enzymes are discussed. It is suggested that each of these two enzymes contain a single anionic site.

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METABOLISM OF PYRROLIDONE CARBOXYLIC ACID BY BACTERIA

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It is well known that glutamic acid (GA) is readily converted to its anhydride, pyrrolidone carboxylic acid (PGA), simply by heating, and is recovered from the latter by acid hydrolysis. In aqueous solution the reaction proceeds reversibly at an acidic and alkaline pH as well (*1*).

It has been supposed for a long time that this reaction might occur also in living organisms. Ratner (*2*) demonstrated the ability of animals to convert D-GA into D-PGA by isolating the latter from the urine after the adminstration of D-GA. Simonart and Yu Chow (*3*) observed the mutual conversion of these two compounds in *Aspergillus oryzae*.

There are several other reports suggesting that PGA can be metabolized in animals (*4–7*). Recently, however, Schultz (*8*) reexamined the earlier studies and demonstrated that L-PGA, when fed to rats, was rapidly hydrolyzed to GA in the acidic medium of stomach, while virtually all of the substance, when administered parenterally, was excreted in the urine without any change.

The present study was undertaken to clarify the metabolic behavior of PGA using a microorganism which can grow on PGA as a sole source of carbon and nitrogen. The results obtained are described in this paper.

EXPERIMENTAL AND METHODS

Chemicals—L-PGA was prepared by the method of Hashizume (*9*) (m. p. 160°). GA-hydrazide and pyrrolydone was kindly supplied from Prof. Akabori and Prof. Murahashi at Osaka University respectively.

Chemical Analysis—L-PGA was hydrolyzed quantitatively to L-GA by heating in 2.5 *N* HCl at 100° for 90 minutes. The resulting L-GA was estimated manometrically using dried cells of *Escherichia coli* Crooks (*10*) as L-GA decarboxylase preparation. Succinic acid was determined by succinic dehydrogenase from pig heart muscle (*11*).

Paper Chromatography: Ascending methods were employed. In the case of amino acids and organic acids (*12*), the solvent mixture, butanol-acetic acid-H_2O (4:1:1), was used. A multiple developing method was also employed to effect good separation of amino acids, if necessary. Unknown spots which were supposed to be peptides were eluted, hydrolyzed with 6 *N* HCl at 100° for 12 hours and rechromatographed to detect their component amino acids. Keto acids: according to Cavallini *et al.* (*13*), butanol saturated with 0.3 per cent ammonia was used as the solvent. Volatile acid: the method of Kennedy and Baker (*14*) was employed.

Manometric Methods—Manometric measurements were carried out by the conventional methods (*11*), unless otherwise stated, at 30° using air as the gas phase.

Isolation of the Microorganisms—Several strains of microorganisms which grow readily in the PGA medium (MI in Table I) were isolated from the soil and purified by succesive platings. One of them which showed the best growth in this medium was used in the present experiments. This microorganism is an aerobic, motile rod and has a polar flagellum. It produces a green fluorescent pigment in the medium. It seems to belong to a genus *Pseudomonas*, but further identification was not performed.

Washed Cells and Dried Cells—Cells grown on the MII medium (Table I) at 30° for 16 hours were harvested by centrifugation, washed with 0.2 per cent KCl and resuspended in the same solution. Dried cells were prepared from a dense washed cell suspension by drying over $CaCl_2$ *in vacuo*.

TABLE I

Composition of Media

Component \ Abbreviation	MI	MII
	%	%
L-PGA	0.5	0.5
KH_2PO_4	0·05	0.05
K_2HPO_4	0.05	0.05
$MgSO_4 \cdot 7H_2O$	0.05	0.05
Peptone	—	0.3
Yeast extract	—	2.0
Meat extract	—	0.3

pH adjusted with NaOH to 7.0 before autoclaving for 20 minutes at 115°.

Cell-Free Preparation—Washed cells were ground with twice their weight of levigated

alumina at 0°. After adding $M/15$ phosphate buffer (pH 7.2), the mixture was centrifuged at 30,000 *g* for 20 min. in the cold and separated into four layers, namely, alumina layer, cell debris, light red particle layer (particle fraction) and clear straw-colored supernatant (soluble fraction). Soluble and particle fractions were mixed and used as the cell-free preparation.

RESULT

Oxidation of PAG—Oxygen uptake and CO_2 evolution by resting cells were measured manometrically. From 2.0 to 2.2 μM of O_2 were consumed and 2.4 to 2.6 μM of CO_2 were evolved per 1 μM of PGA, so the approximate RQ value of 1.3 was found (Fig. 1).

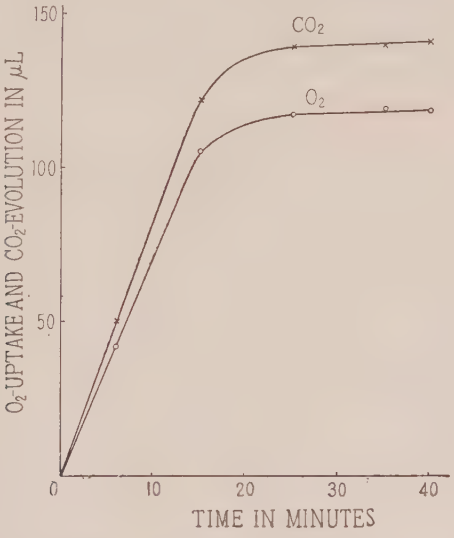

Fig. 1. Oxidation of PGA by resting cells.
Composition of the reaction mixture: Na-PGA 3 μM, $M/10$ phosphate buffer (pH 5.5) 1.0 ml., cells 7 mg. dry wt., Total volume, 1.7 ml. Temp., 30°. Gas phase, air. Data are corrected for endogenous respiration.

GA, α-ketoglutaric acid, succinic acid and proline were also oxidized immediately by the washed cells. Dried cells could also oxidize PGA, GA, α-ketoglutarate, succinate and proline, although the activities to oxidize these substances were lower as compared with those of washed

cells. Relative values of Q_{O_2} of the substances mentioned above are listed in Table II.

CO_2 evolution in the anaerobic decomposition of PGA and GA was measured manometrically under nitrogen as the gas phase. Chemical determination was also performed to detect the decomposition of these compounds. Both washed and dried cells, which were obtained by either shaking or stationary culture, were used in this experiment. In either case little decomposition of these substances could be observed under anaerobic conditions.

Examination of Substances Produced during the Aerobic Degradation of PGA—In order to obtain some information about the metabolic pathway of PGA, the oxidation products were examined using washed cell suspensions. The reaction was carried out under the air as the gas phase in a closed 500 ml. shaking flask with a side arm containing 3 ml. of 2 N H_2SO_4. The composition of the reaction mixture was as follows:

TABLE II

Q_{O_2} of Washed Cells and Dried Cells for Various Substrates

Substrate	Q_{O_2}	
	Washed cells	Dried cells
L-PGA	100	11
L-GA	50	13
α-Ketoglutarate	40	13
Succinate	105	12
Proline	50	10

Q_{O_2} are expressed as μl. oxygen taken up per hour per mg. dry wt. of cells.

M/15 phosphate buffer (pH 7.2) 25 ml., cell suspension 10 ml. (370 mg. dry wt.) and 0.25 M Na-PGA 5 ml. In the control experiment 5 ml. of H_2O was added in place of PGA. Total volume, 40 ml. The flasks were shaken at 30°. After 2 hours H_2SO_4 was added from the side arm to the medium to stop the reaction and to absorb ammonia which might have been evolved. Reaction process was followed manometrically and about 0.7 mole. of O_2 uptake per 1 mole of PGA was observed in this aerobic reaction. Cells were separated from the medium by centrifugation. PGA and GA were determined on the supernatants. Total N determination was performed both on the cell fraction and on

the supernatant. Nitrogen balance showed that about one fourth of consumed PGA was incorporated into cell materials. Volatile acids were separated by steam distillation and titrated with $N/100$ NaOH. In this volatile acid fraction, only acetic acid was detected by paperchromatography, so the titrated value showed the amount of acetic acid. Another part of reaction mixture was extracted with acidic ether and organic acids and keto acids in the extract were examined by paperchromatography. Several organic acids were detected, among which succinate was the main component. These results were summarized in Table III.

Oxidation of PGA by the Cell-free Preparation—Particle and soluble fractions were examined for their activity of the oxidation of PGA and GA. The results were as follows: The soluble fraction consumed 1.2 μM of PGA and 6.8 μM of GA, and the particle fraction 5.6 μM of PGA and 20 μM of GA per hour per mg. N of the preparations. However, these values fluctuate more or less from one preparation to another.

TABLE III

Oxidative Decomposition of PGA by Washed Cells

N balance	
Total N found in supernatant	1537 μM
NH_3-N	1076
Total N incorporated into cell material	458
Total N recovered	1995
Total N added as substrate	2000
L-PGA remained	28
L-GA produced	220
Acetic acid	47
Succinic acid	70
Substances detected by paperchromatography	
Non-volatile organic acids: Succinate, lactate, PGA, malate, unknown spot with Rf 0.22 and 0.15.	
Volatile acid: Acetate.	
Keto acids: α-Ketoglutarate (Rf 0.16), pyruvate (Rf 0.42), unknown spot with Rf 0.11.	
Amino acids: GA, alanine, aspartate, peptide 1 (Component amino acid is GA, peptide 2 (Components amino acid are GA and alanine), unknown spots with Rf 0.45, and 0.55.	

In any way, the activity of oxidizing PGA existed mostly in the particle fraction. The cell-free preparation took up about 4.5 μM O_2/μM PGA or GA, indicating the complete oxidation of these substances. The activity of the preparation to oxidize PGA was completely abolished by its incubation at 45° for ten min., while a large proportion of activity of oxidizing GA still remained.

Effect of Inhibitors—As the ratio of the activity of the PGA oxidation to that of GA oxidation varied according to the method of preparation, it was supposed that PGA and GA were decomposed by different enzyme systems at the first stage. The separation of two systems was attempted by examination of the effect of various inhibitors on these systems. *M*/1000 cyanide inhibited 90 per cent of the oxidation of PGA by washed cells, while about 30 per cent of that of GA. *M*/33 GA-γ-hydrazide inhibited the oxidation of GA by cell-free preparation almost completely, whereas inhibition was scarcely recognized in the case of PGA oxidation (Fig. 2). These results strongly indicate that main path of the oxidative decomposition of PGA is not *via* GA.

Oxidation of Pyrrolidone—As the first step of decomposition of PGA seems not to be direct hydrolysis to GA as described above, it could be supposed that PGA may be metabolized via pyrrolidone, Q_{O_2} for pyrrolidone by washed cells was about 10, a tenth of that for PGA. The cell-free preparation, which oxidized PGA completely, could not oxidize pyrrolidone at all. Therefore pyrrolidone is not likely to be an intermediate in the decomposition of PGA.

DISCUSSION

It has been clearly demonstrated that PGA can be utilized and metabolized aerobically by this bacterium. The quantitative experiment on the decomposition of PGA showed that GA, succinate, acetate and many other organic compounds are produced from this compound.

As to the metabolic pathways of PGA to enter the usual respiratory cycle, there are three possible pathways in this microorganism (Fig. 3). Results from the inhibition experiments with GA-hydrazide indicate that the path I is not the main, while the path III is unlikely because pyrrolidone is not oxidized by cell-free preparation which oxidized PGA completely. Therefore the path II remains as the most probable pathway. According to this scheme PGA is oxidized to pyroglutaramic acid and then converted, *via* α-ketoglutaramic acid, to succinic acid or to α-ketoglutaric acid. According to the recnt studies of Meister

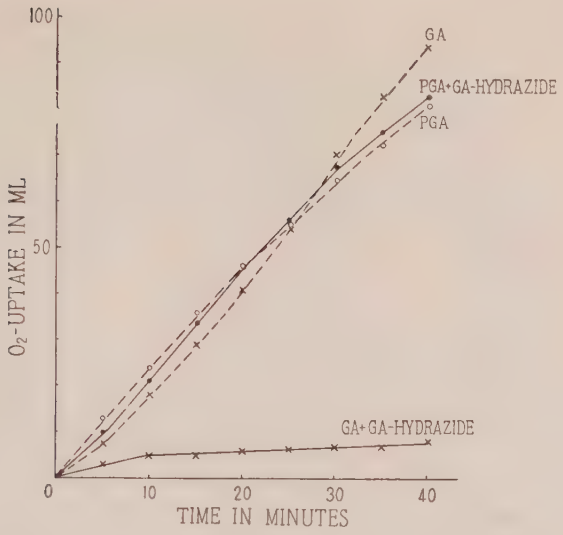

FIG. 2. Effect of GA-hydrazide on the oxidation of PGA and GA by cell-free preparation.

Composition of the reaction mixture: Na PGA *M*/700, phosphate buffer (pH 7.0) *M*/20, GA-hydrazide *M*/33, cell free preparation 12 mg. dry wt. Total volume, 2.0 ml. Temp., 30°. Gas phase, air.

Data are corrected for endogenous respiration.

(*15*), α-ketoglutaramic acid plays an important role in the normal metabolism of animal cell as an intermediate of the transamination of glutamine. Among the decomposition products of PGA by washed cells, authors found an unknown keto acid in addition to α-ketoglutarate by paperchromatography. Though this substance was not yet conclusively identified, it may give some clue on this problem. Anyhow further studies are required.

The interconversion between GA and PGA in living cells is sometimes suggested. In the present study the hydrolysis of PGA to GA is excluded as the main path of the oxidation of PGA. The reaction from GA to PGA is also unlikely from the following experimental results: (1) Some cell-free preparation, which did not show any activity for PGA, still oxidized GA strongly; (2) the incubation of cell free preparation at 45° for 10 minutes almost completely abolished the activity for PGA, while that for GA is scarcely diminished; (3) *M*/1000 cyanide

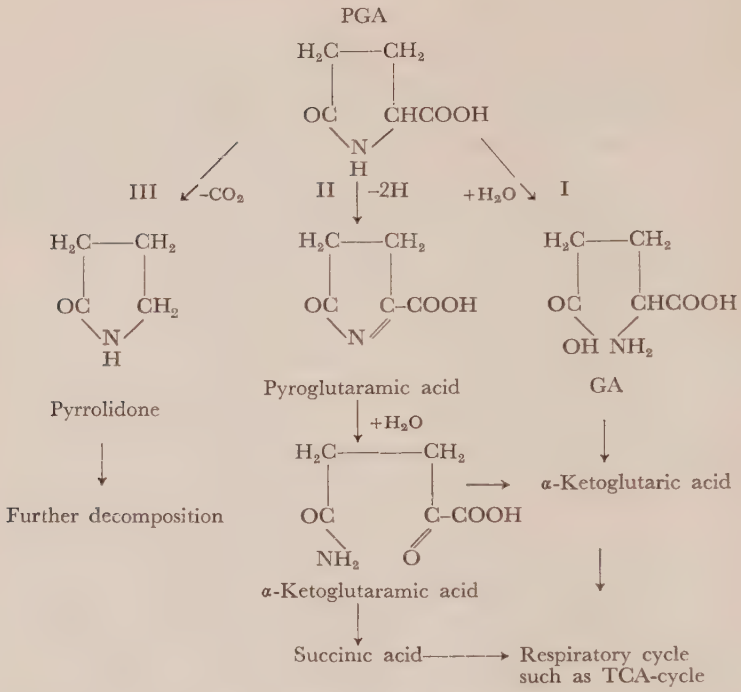

FIG. 3. The possible pathways of the aerobic decomposition of PGA.

inhibited the oxidation of PGA almost completely, while the oxidation of GA was inhibited only about 30 per cent. Therefore it seems that the interconversion between GA and PGA do not take place in the authors' bacterium.

SUMMARY

Several strains of microorganisms, which grow well on the medium containing pyrrolidone carboxylic acid as a sole source of carbon and nitrogen, have been isolated from soil.

Using one of them, aerobic decomposition of pyrrolidone carboxylic acid was studied. As the decomposition products by washed cell suspension, glutamic acid, succinic acid, acetic acid and other organic compounds were detected.

The cell-free preparation, which can oxidize PGA completely, was obtained from this organism.

The first stage of the oxidation of PGA was concluded to be neither hydrolysis to GA nor decarboxylation to pyrrolidone.

A possible pathway (*via* pyroglutaramic acid) was discussed.

The authors wish to express their sincere thanks to Prof. S. Akabori, Prof. S. Funahashi and Dr. B. Maruo for their kind guidance and many useful suggestions throughout the present work.

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OXIDATION OF LACTATE BY CORYNEBACTERIUM DIPHTHERIAE ADAPTED TO IRON-DEFICIENT MEDIA

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In the previous paper (*1*), it was shown that *C. diphtheriae* cultivated in the iron-deficient condition, which was essential for the formation of the toxin by this bacterium, synthesized larger amount of flavin compound than those in an iron-sufficient medium. Flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) were found in the bacterial cells cultivated in the iron-deficient medium, into which free riboflavin (FR) and FMN had been excreted. Further research (*2*) ascertained that the amount of iron in a culture medium determined the level of cytochrome formed in the cells. However, the endogenous respiration of *C. diphtheriae* cultivated in the iron-deficient medium showed an activity similar to that in the iron-sufficient one, though the latter was much more sensitive to cyanide or azide than the former.

Based on these facts, a working hypothesis was adopted that *C. diphtheriae* cultivated in the iron-deficient condition increased or formed some autoxidizable flavin enzymes.

Moreover, a work (*3*) concerning the oxidation of several organic acids by *C. diphtheriae* revealed that lactate is the most utilizable organic acid under both conditions.

In this paper, the oxidation of lactate by *C. diphtheriae* in an iron-deficient medium is described comparing with those in an iron-sufficient medium.

METHODS AND MATERIALS

Basal medium (called " Medium II " in this paper) was prepared as follows: Equal volumes of the tryptic digest broth [Pope's media (*4*)] and the hog stomach digest broth [Taylor's media (*5*)] were

mixed and iron was removed by calcium phosphate gel. By this medium, the I.f value reached 170 after 38 hours of shaking culture.

Medium II contained about 0.1 γ/ml. of Fe^{++}. Medium I was prepared from Medium II by the addition of $FeSO_4 \cdot 7H_2O$ so as to contain 20 γ/ml. of Fe^{++}. Medium III was the same as Medium II except that it contained 6×10^{-5} *M* of α, α′-dipyridyl in the final concentration.

C. diphtheriae of Park Williams No. 8 strain was used. For the cultivation, shaking culture method (*6*) was adopted and the bacteria were harvested after 16–19 hours' cultivation, in which time the highest activity for the oxidation of lactate was obtained.

The bacteria were centrifuged down and washed three times with saline solution and were then made resting cells. These cells were placed in the main chamber of a Warburg manometer with DL-lactate (*M*/20 in the final concentration) and phosphate buffer of pH 6.5 (*M*/50 in the final concentration).

To determine the substance produced from lactate, paper chromatographic method was adopted. Keto acids were examined by paper chromatography of their 2,4-dinitrophenylhydrazones using *n*-butanol as the mobile phase (*7*). Fatty acids were examined by paper chromatography of their hydroxamic acids (*8*).

EXPERIMENTALS AND DISCUSSIONS

The Mode of Oxidation of Lactate by C. diphtheriae Cultivated in an Iron-Deficient or Iron-Sufficient Medium—Oxidation of lactate by the cells cultivated in the above-mentioned media and their inhibition by several inhibitors were examined. As inhibitors, each of cyanide, azide, Ag ions, Cu ions, and pyruvate was added from the side arm of a Warburg flask, and their final concentrations were 5×10^{-3}, 5×10^{-3}, 3×10^{-4}, 3×10^{-4} and 1.7×10^{-2} *M* respectively.

By the addition of each of cyanide, azide and pyruvate, oxygen consumption by the cells in Medium I was inhibited, and that by the cells in Medium II was slightly inhibited, while that by the cells in Medium III remained insensitive to the addition of these inhibitors as shown in Fig. 1.

It was common to all cases of these three media that the oxidation of lactate by the cells was completely inhibited by each of Ag and Cu ions as also shown in Fig. 1.

From these results it was considered that the removal of iron from

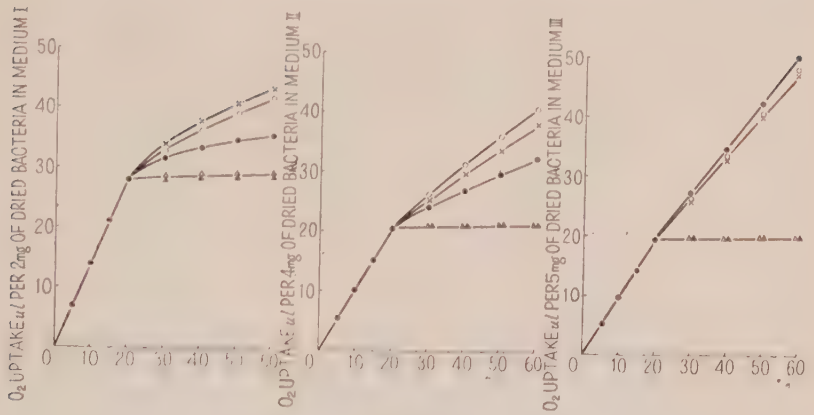

FIG. 1. Oxidation of lactate by *C. diphtheriae* cultivated in iron-sufficient or -deficient media, and their inhibition by several inhibitors.

Main chamber contained M/20 DL-lactate and *M*/50 phosphate buffer (pH 6.5) in their final concentration. Twenty minutes after the reading, inhibitors were mixed.

Inhibitors and their final concentration: ●—● KCN, $5\times10^{-3}M$; ○—○ azide, $5\times10^{-3}\,M$; ▲—▲ Ag ion, $3\times10^{-4}\,M$; △—△ Cu ion, $3\times10^{-4}\,M$; ×—× pyruvate, $1.7\times10^{-2}\,M$.

culture medium changed the manner of the oxidation of lactate by *C. diphtheriae*. In the case of a medium containing only few amount of iron, lactate may be converted into some substance other than pyruvate.

The oxygen-uptake and the evolution of carbon dioxide were examined. As shown in Table I, there was only little amount of carbon dioxide evolution in the case of Medium I. The molecular ratio of oxygen uptake to evolution of carbon dioxide was nearly 1 in the case of Medium III.

The flavin enzyme which oxidizes lactate to acetate has already been reported by Edson (*9*) and Yamamura *et al.* (*10*), and in our experiments the manner of oxidation of lactate by *C. diphtheriae* in the case of Medium III was assumed to be similar to those by the lactic oxidases reported by them.

So, to determine the substance produced from lactate, paper chromatographic methods were adopted. Pyruvate was clearly demonstrated in the case of Medium I, and acetate was clearly found in the case of Medium III. In the case of Medium II, both pyruvate and

TABLE I

Uptake of Oxygen and Evolution of Carbon Dioxide in the Oxidation of Lactate by C. diphtheriae

	Q_{O_2}	Q_{CO_2}
Cells in Medium I	35.0	2.0
,, ,, ,, II	15.0	8.5
,, ,, ,, III	12.2	11.5

acetate were observed.

Experiments for the Cell-Free Extract of C. diphtheriae Cultivated in an Iron-Deficient Medium—To study further the lactic oxidase formed by *C. diphtheriae* cultivated in an iron-deficient medium, cell-free extract was prepared and examined. From the cells cultivated in Medium III, cell-free extract was prepared by the following procedures: The cells washed once with distilled water were lyophilized to dryness. One g. of lyophilized cells was mixed with 5 ml. of phosphate buffer (pH 6.5), and was ground with glass powder in a glass homogenizer maintained at near 0° by methanol and solid carbon dioxide. The homogenate was diluted to 10 ml with the same phosphate buffer, shaken, and then centrifuged for 20 minutes at 10,000 r.p.m. The supernatant was used as the cell-free extract.

The cell-free extract was tested in a Warburg manometer with the following reaction mixture: One ml. of the cell-free extract was placed in the main chamber of the flask, and 0.2 ml of *M* DL-lactate and 20 γ of FAD (final concentration being 1.3×10^{-5} *M*) or equimolecular FMN or FR in the side arm. The whole volume of the liquid was then made up to 2.0 ml with *M*/10 phosphate buffer (pH 6.5). Oxygen uptake per 1.0 ml of the cell-free extract in this reaction mixture is shown in Fig. 2.

As shown in Fig. 2. oxygen uptake by this extract without lactate was negligible. Lactate was oxidized by this extract, and the addition of FAD was effective to promote the oxygen uptake, while that of FMN or FR was ineffective. The oxidation of lactate in this reaction mixture was insensitive to pyruvate addition. Pyruvate added instead of lactate was not oxidized in this reaction mixture.

To promote the oxygen uptake by this reaction mixture, coexistence of one or all of diphosphopyridine nucleotide (DPN), cytochrome *c* and Mg ion was ineffective as shown in Fig. 3.

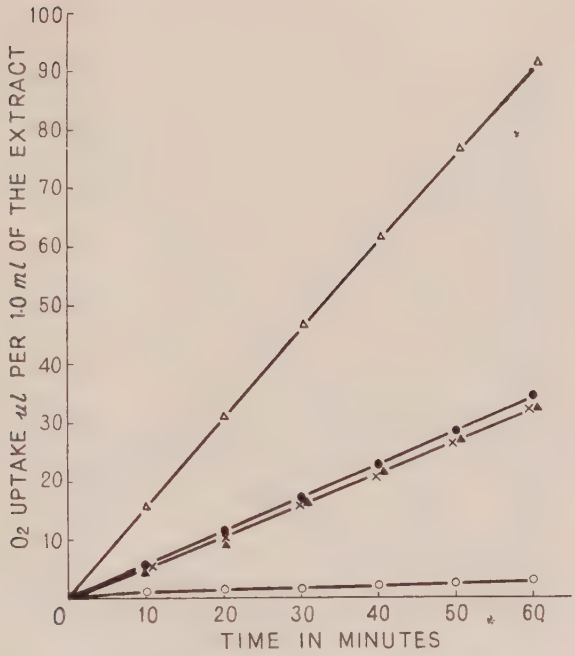

FIG. 2. Oxidation of lactate by the cell-free extract of *C. diphtheriae* cultivated in an iron-deficient medium, and the effects of added flavins.

Main chamber contained 1.0 ml. of the cell-free extract and $M/10$ DL-lactate (final concentration) in phosphate buffer of pH 6.5.

Flavins added and their final concentrations: △—△ FAD, 1.3×10^{-5} M; ▲—▲ FMN, 1.3×10^{-5} M; ×—× FR, 1.3×10^{-5} M; ●—● without the addition of flavins.

○—○ shows the oxygen uptake of the cell-free extract.

Oxygen uptake in this reaction mixture was completely inhibited both by Ag and Cu ions in their final concentration of 2×10^{-4} and 1×10^{-4} M, respectively, while it was not inhibited either by cyanide, azide, hydroxylamine, or pyrurate in their final concentration of 5×10^{-3}, 5×10^{-3}, 3.3×10^{-4}, and 1.7×10^{-2} M, respectively as shown in Fig. 4.

Oxidation product from lactate in this reaction mixture was also considered to be acetate from the result of paper chromatography and the RQ of the reaction which was nearly 1.0.

From these results it was considered that *C. diphtheriae* cultivated

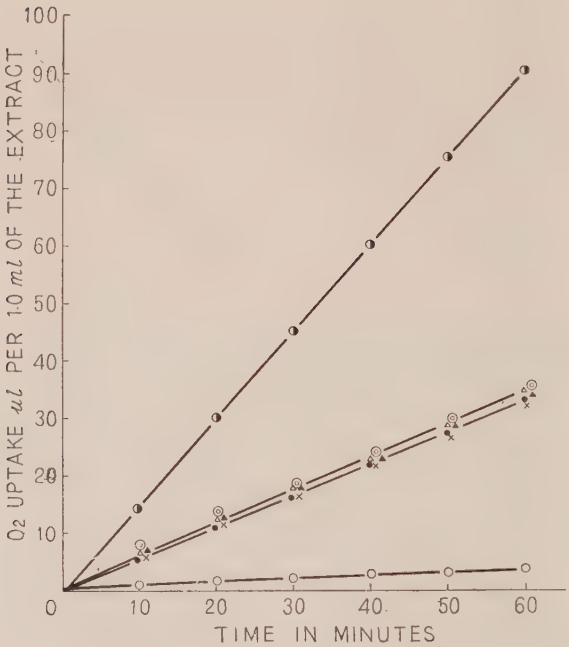

FIG. 3. Oxidation of lactate by the cell-free extract of *C. diphtheriae* cultivated in an iron-deficient medium, and the effects of several substances added.

Main chamber contained 1.0 ml. of the cell-free extract and *M*/10 DL-lactate (final concentration) in phosphate buffer of pH 6.5.

Added substances and their final concentrations: △—△ DPN, 5×10^{-4} *M*; ▲—▲ cytochrome c, 1×10^{-5} *M*; ×—× Mg ion, 1×10^{-4} *M*; ◎—◎ DPN, cytochrome c, and Mg-ion, above-mentioned concentration; ◑—◑ DPN, cytochrome c, Mg ion (same as above), and 1.3×10^{-5} *M* of FAD.

○—○ shows the oxygen uptake of the cell free extract.

in an iron-sufficient medium contained the lactic oxidase which required cytochrome system and oxidized lactate to pyruvate, while those cultivated in an iron-deficient one contained another lactic oxidase which required only FAD as a coenzyme and oxidized lactate to acetate. The latter lactic oxidase is similar to that found in *Mycobacterium phlei* by Edson (*9*), and in *Mycobacterium tuberculosis avium* by Yamamura *et al.* (*10*).

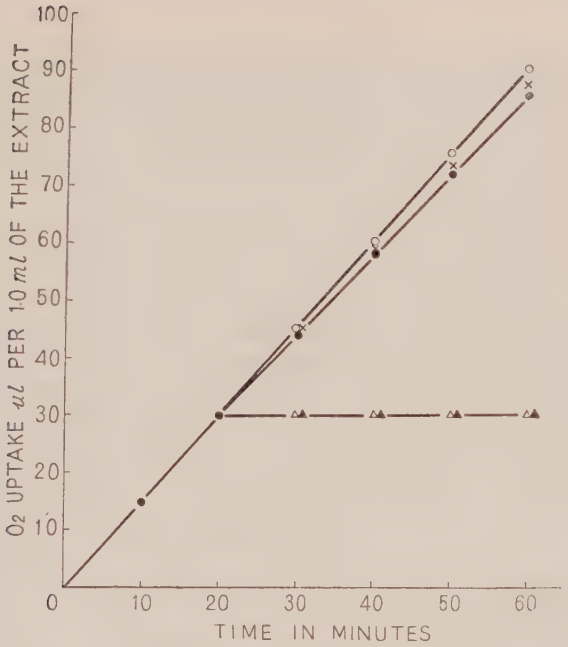

FIG. 4. Oxidation of lactate by the cell-free extract of *C. diphtheriae* cultivated in an iron-deficient medium, and the effect of several inhibitors added.

Main chamber contained 1.0 ml. of the cell-free extract, $M/10$ DL-lactate (final concentration), and 1.3×10^{-5} M of FAD in phosphate buffer of pH 6.5.

Added inhibitors and their final concentrations: ●—● KCN, 5×10^{-3} M; ○—○ azide, 5×10^{-3} M, or hydroxylamine, 3.3×10^{-4} M; ▲—▲ Ag ion, 2×10^{-4} M; △—△ Cu ion, 1×10^{-4} M; ×—× pyruvate, 1.7×10^{-2} M.

Further, it was ascertained that *C. diphtheriae*, which contains the latter lactic oxidase, can easily be made to contain the former lactic oxidase by the addition of iron to the medium, and *vice versa*.

These facts show that *C. diphtheriae* forms this lactic oxidase in order to adapts itself to the iron-deficient condition, and it is suggested that this bacterium, when iron system becomes decreasingly available, adapts itself to the condition by synthesizing autoxidizable flavin enzymes.

SUMMARY

1. The oxidation of lactate by *C. diphtheriae* cultivated in an iron-deficient medium was not inhibited by cyanide, azide and pyruvate, while that in an iron-sufficient medium was inhibited by these inhibitors. By Ag and Cu ions, both were inhibited seriously. In the oxidation product of the former case, acetate was found, while in that of the latter, pyruvate was demonstrated.

2. The oxidation of lactate by the cell-free extract of *C. diphtheriae* cultivated in an iron-deficient medium were accelerated by the addition of FAD, and not by FMN, free riboflavin, DPN, cytochrome c and Mg ion. The oxygen uptake were inhibited by Cu and Ag ions, but not by cyanide, azide or hydroxylamine.

From these facts, it will be considered that the lactic oxidase formed by *C. diphtheriae* adaptively to the iron-deficient condition was an autoxidizable flavin enzyme which catalyzes the reaction from lactate to acetate.

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STUDIES ON HISTIDINE DEAMINASE*,**

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(Received for publication, Eebruary 25, 1956)

Ichihara *et al.* (*1*) have reported, that purified histidine deaminase of rabbit liver regained its activity by the addition of folic acid and SH-glutathione. Suda *et al.* (*2*) purified histidine deaminase of soil bacteria and demonstrated restoration of activity by the addition of Hg^{++}, Cd^{++} or Zn^{++}. According to their recent publication (*3*), purified histidine deaminase of guinea pig liver regained activity by the addition of Cd^{++} and SH-compounds (SH-glutathione, thioglycollate or cysteine). Histidine deaminase of cat liver was inactivated completely by EDTA (ethylendiamine-tetraacetate) (*4*) and the lost activity was restored by Mg^{++}, Mn^{++} and Ca^{++} (*5*). The authors repeated their previous experiments on histidine deaminase with regards to metal ions.

EXPERIMENTAL

Materials

Ca-Phosphate Gel—This was prepared by the method of Tsuchihashi (*6*) from Na_3PO_4 and $CaCl_2$(Merck).

EDTA—This preparation was kindly provided by Prof. J. Kawamata.

Folic Acid and Folinic Acid—These preparations were made available by the Takeda Pharmaceutical Ind. Ltd.

SH-Glutathione—This was a preparation of the Ishizu Pharmaceutical Ind. Ltd.

Enzyme Preparations—L-Histidine monohydrochloride (0.5 g. per kg. of body weight) was administered to the rabbit by means of a stomach tube and after 6 hours the animal

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* The compendium of this work was announced at the 2nd Kinki Local Meeting of the Japanese biochemical Society on 5 June, 1954.

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was sacrified by bleeding. The liver, ground with an equal amount of sea sand, was extracted with 5 times of water at 0–5° for one hour and centrifuged at 300 r.p.m. for 20 minutes. The supernatant was adjusted to pH 5.2 with 4 per cent acetic acid and centrifuged at 3000 r.p.m. for 30 minutes. The precipitate, dissolved with minimal distilled water, was adjusted to pH 7.2 with 4 per cent sodium bicarbonate and fractionated with saturated ammonium sulfate. The 30–50 per cent fraction was dissolved in a quantity of chilled water equal to the amount of the liver (Salting out enzyme preparation). After Ca-phosphate-gel, corresponding to 4 per cent of the enzyme solution, was added, the suspension was kept at 0–5° for 30 minutes and centrifuged at 3000 r.p.m. for 10 minutes. After the adsorbent was washed with chilled water until Nessler's and Sera's reactions (*7*) were negative, it was eluted with the same volume of *M*/10 phosphate buffer (pH 8.3) as that of the enzyme solution before adsorption at 0–5° for 24 hours. The eluate was centrifuged at 3000 r.p.m. for 10 minutes and the supernatant which separated from the adsorbent was used as the purified enzyme preparation.

Methods

Determination of Histidine—Histidine was determined photometrically after Sera's reaction (*7*), which is based on the bromine reaction, using S_{53} filter and the 10 mm cubette.

Determination of Ammonia—Ammonia was determined by the method of Archibald (*8*) using the S_{47} filter and the 20 mm cuvette.

Paperchromatography—The developing solvent was a mixture of acetic acid, butanol and water (1:4:1).

Condition of the Enzyme Reaction—The reactive solution was composed of 1.0 ml. of enzyme solution, 1000 γ of histidine monohydrochloride H_2O, 0.3 ml. of 1 *M* phosphate buffer (pH 8.3) and the supplements. The total volume of the solution was made up to 3.0 ml. with distilled water. The reaction was carried out at pH 8.3 and 37.5° for 3 hours. After the reaction, 1.0 ml. of 30 per cent trichloroacetic acid and 1.0 ml. of distilled water were added and the solution was filtered. 1.0 ml. of the filtrate was used for histidine determination.

RESULTS

Experiment on the Enzyme Salted out—As shown in Fig. 1, the activity of the salting out histidine deaminase was completely inhibited by 10^{-4} *M* of EDTA and as shown in Table I, the activity, inactivated by EDTA, was restored by the addition of Co^{++}, Mn^{++}, Cd^{++} or Zn^{++}, most effectively by Co^{++}.

Experiment on the Purified Enzyme—As Table II shows, the purified preparation of histidine deaminase was completely inactive and regained its activity by the addition of folic acid and SH-glutathione as previously

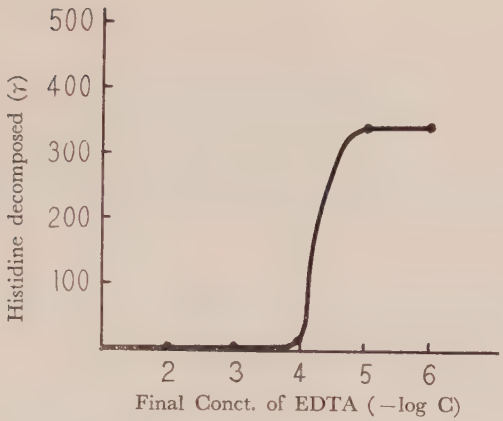

FIG. 1. Inhibitory effect of EDTA.

The enzyme preparation decomposed 340 γ of histidine without EDTA.

TABLE I

Restoration of Enzyme Activity Inactivated by EDTA

Histidine decomposed (γ)	Additions					
	None	Co	Mn	Zn	Cd	Mg
	0	740	570	610	550	0

Condition of reaction: 0.1 ml. of 10^{-2} *M* $CoCl_2$, $MnSO_4$, $ZnSO_4$, $CdSO_4$ and $MgSO_4$ (final conct. 3.3×10^{-4} *M*); 0.6 ml. of 10^{-3} *M* EDTA (final conct. 2×10^{-4} *M*).

Notice: The enzyme preparation decomposed 640 γ of histidine without EDTA.

reported (*1*). The restorating effect was demonstrated neither by single addition of folic acid or folinic acid nor by folinic acid or SH-glutathione.

When EDTA was added (final conct. 2×10^{-4} *M*) prior to salting out the enzyme and kept at 37.5° for 10 minutes and the enzyme then purified as described above, the inactive enzyme was reactivated only by the addition of folic acid, SH-glutathione and Co^{++}. Folinic acid, SH-glutathione and Co^{++}, however, have no such effect (Tables III and IV).

TABLE II

Effect of Additions on the Activity of Purified Enzyme

(without addition of EDTA)

	Additions				
Decomposition of histidine (γ)	None	Folic acid	Folinic acid	Folic acid SH-glutathione	Folinic acid SH-glutathione
	0	0	0	170	0

Condition of reaction: 500 γ/0.5 ml. of folic acid (final concn. 3.8×10^{-4} M); 500 γ/0.5 ml. of SH-glutathione (final concn. 0.6×10^{-3} M); 500 γ/0.5 ml. of folinic acid (final concn. 3.3×10^{-3} M).

Notice: The salting out enzyme preparation decomposed 250 γ of histidine.

TABLE III

Effect of Additions on the Restoration of Activity of Purified Enzyme Previously Treated with EDTA

	Enzyme satled out	Additions	Bivalent metal ions added						
			None	Co	Zn	Mn	Cd	Mg	Fe
Histidine decomposed (γ)	340	——		0	0	0	0	0	/
		Folic acid, SH-glutathione	0	340	0	0	0	0	0
		Folinic acid, SH-glutathione	0	0	/	/	/	/	/

Condition of reaction: 0.1 ml. of 10^{-2} M each metal ion (final concn. 3.3×10^{-4} M); 1000 γ/0.5 ml. of folic acid (final concn. 7.6×10^{-4} M); 500 γ/0.5 ml. of folinic acid (final concn. 3.3×10^{-3} M); 1000 γ/0.5 ml. of SH-glutathione (final concn. 1.1×10^{-3} M).

Determination of Ammonia Formed—The reaction was carried out using the vessel with stopper and side arm, in which 1.0 ml. of 1 N N_2SO_4 was contained. After the reaction, the contents of main compartment and side arm were mixed and 1.0 ml. of 30 per cent trichloroacetic acid was added. 2 ml. of the filtrate was used for the determination of ammonia as described. As shown in Table V, 1 M ammonia was formed from 1 M histidine by the enzyme, activated by the addition of folic

acid, SH-glutathione and Co# (Table V).

TABLE IV

Effect of Folic Acid, SH-Glutathione and Co Ion on the Purified Enzyme Treated Previously with EDTA

Co# (3.3×10^{-4} *M*)	Folic acid (7.6×10^{-4} *M*)	SH-glutathione (1.1×10^{-3} *M*)	Histidine decomposed (γ)
+	−	−	0
−	+	−	0
−	−	+	0
+	+	−	150
+	−	+	280§
+	+	+	350

§ This preparation was purified from the salting out enzyme, which decomposed 400 γ of histidine.

Conditions are the same as above described. By the enzyme salted out 420 γ histidine was decomposed.

TABLE V

Ammonia Formed from Histidine by the Inactivate Purified Enzyme after Folic Acid, SH-Glutathione and Co Ion Were Added

Exper. No.	Histidine decomposed (γ)	Ammonia formed (γ)
1	320 (1.55 *M*)	24.9 (1.46 *M*)
2	320 (1.55 *M*)	25.9 (1.52 *M*)

The condition of reaction was the same as in Table III.

Paperchromatography—The enzyme, previously treated with EDTA and adsorbed by Ca-phosphate gel, was used with addition of folic acid, SH-glutathione and Co#. After the reaction, methanol was added (final concentration: 70 per cent) for deproteinization. The filtrate was evaporated in vacuum and the concentrate was developed by means of paperchromatography. Urocanic acid was demonstrated by Pauly's diazo reaction (urocanic acid: Rf 0.53) and by the ninhydrine reaction glutamic acid was not proved.

Experiment with Dialyzed Enzyme—The purified enzyme solution, prepared as described above, was dialyzed against 1000 times of distilled water at 0–5° for 18 hours. The fraction precipitated by ammonium

sulfate at 70 per cent saturation was centrifuged at 3000 r.p.m. for 25 minutes. The precipitate was dissolved in equal amount of water as that of the started enzyme solution and used for the following experiments. As shown in the next Table, the enzyme thus obtained was completely inactive and the activity was restored most effectively by the addition of all three, folic acid, SH-glutathione and Co^{++}. While the combined additions of folic acid with Co^{++} as well as SH-glutathione with Co^{++} have considerable effect, the single addition of each and the combined addition of bivalent metal ions other than Co^{++} with folic acid and SH-glutathione have no effect on the restoration of the enzyme activity.

TABLE VI

Effect of Folic Acid, SH-Glutathione and Co Ion etc. on the Restoration of Enzyme Activity

Folic acid	SH-glutathione	Co	Ca	Mg	Zn	Mn	Fe	Cd	Histidine decomposed (γ)
−	−	−	−	−	−	−	−	−	0
+	−	−	−	−	−	−	−	−	0
−	+	−	−	−	−	−	−	−	0
+	+	−	−	−	−	−	−	−	0
+	−	+	−	−	−	−	−	−	100
−	+	+	−	−	−	−	−	−	320
+	+	+	−	−	−	−	−	−	360–380
+	+	−	+	−	−	−	−	−	0
+	+	−	−	+	−	−	−	−	0
+	+	−	−	−	+	−	−	−	0
+	+	−	−	−	−	+	−	−	0
+	+	−	−	−	−	−	+	−	0
+	+	−	−	−	−	−	−	+	0

Notice: By the salting out enzyme 420 γ of histidine was decomposed. Condition of the reaction: 1000 γ/0.5 ml. of folic acid (final conct. 7.6×10^{-4} *M*); 1000 γ/0.5 ml. of SH-glutathione (final conct. 1.1×10^{-3} *M*); 0.1 ml. of 10^{-2} *M* each bivalent metal ion (final conct. 3.3×10^{-4} *M*).

Saturation of Folic Acid—In the condition, described in Fig. 2, the saturation of folic acid lied at 10–20 γ.

DISCUSSION

As Borek *et al.* (*4*) and Mehler *et al.* (*5*) have reported, histidine deaminase obtained by them was inactivated by EDTA and reactivated

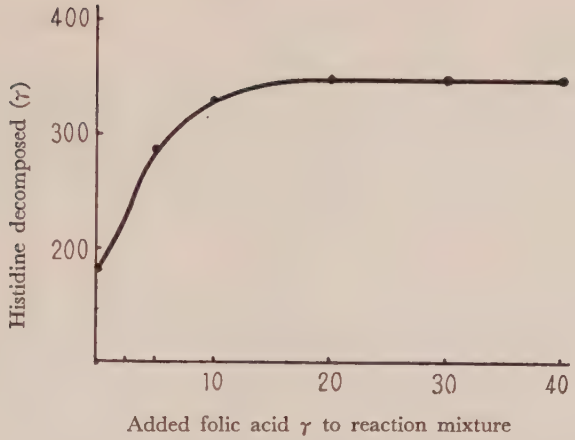

FIG. 2. Saturation curve of folic acid for the purified enzyme in the presence of SH-glutathione and Co ion.

Notice: This preparation was purified from the salting out enzyme which decomposed 359.6 γ of histidine, and decomposed 183.3 γ of histidine by the addition of Co++ and SH-glutathione without folic acid.

Condition of reaction: 1.78 mg./1.0 ml. of histidine (10 μM); 0.3 ml. of each concentration of folic acid; 1000 γ/0.3 ml. of SH-glutathione (final conct. 1.1×10^{-3} *M*); 0.1 ml. of 10^{-2} *M* $CoCl_2$ (final conct. 3.3×10^{-4} *M*).

by bivalent metal ions (Table I). The purified enzyme, treated previously with EDTA, however, did not regain its activity by addition of metal ion with the exception of Co++, even in the presence of folic acid and SH-glutathione (Tables, III, IV and VI). The authors repeated the previous experiment (*1*) and confirmed that the purified histidine deaminase recovered its activity by the addition of folic acid and SH-glutathione (Table II). The enzyme was reactivated considerablly by the addition of two of the followings, folic acid, SH-glutathione or Co++ but most effectively by all three (Tables IV and V).

Folinic acid was effective (*9* and *10*) as donor of the formyl group, but it was found to be ineffective either by single addition or even by combination with SH-glutathione and Co++ ion (Tables II and III).

As a metabolite of histidine, urocanic acid was proved by means of paperchromatography, but glutamic acid not all. One molecule of ammonia was formed from one molecule of histidine by histidine de-

aminase reactivated by the addition of folic acid, SH-glutathione and Co^{++} (Table V).

From the results above mentioned, it can be concluded, that SH-glutathione and folic acid are essential for histidine deaminase. The results of Tables I, III, IV, and VI can be explained by the presence of Co^{++} in the enzyme preparation. As the metal chelater, EDTA is more sensitive to the other metal ions than to Co^{++} (*11*) and the Co^{++}, combined to EDTA, might be freed by other metals and become effective (Table I).

Just before the copy was sent to the Editorial Board of this Journal, the report of Kato *et al.* (*12*) was published, which differed fundamentally from the authors' results. Their enzyme preparation was reactivated by the addition of SH-compounds and Cd^{++} but folic acid and Co^{++} were entirely effectless. There were two chief differences between the both experiments; Kato *et al.* used the guinea pig and the authors used the rabbit and the former heated the liver extract at 65° for 8 minutes in the first step of the enzyme preparation. Hereupon Koizumi, one of the present authors, purified the histidine deaminase

TABLE VII

Effect of Folic acid, SH-Glutathion, Co and Cd Ion on the Histidine Deaminase, Purified by the Procedure II of Kato et al.

Additions				Histidine decomposed (γ)
Co (3.3×10^{-4} *M*)	Cd ($3.3\times10^{-}$ *M*)	Folic acid (1.52×10^{-4} *M*)	SH-glutathione (1.1×10^{-3} *M*)	
−	−	−	−	0
−	−	−	+	0
+	−	−	+	85.6
−	+	−	+	0
−	−	+	+	0
+	−	+	−	85.6
−	+	+	−	0
+	−	+	+	663.4
−	+	+	+	0

Notice; This preparation was purified from the isoelectrically precipitated enzyme which decomposed 791.8 γ of histidine.

Condition of the reaction: 2.14 mg./1.0 ml. of histidine (10 μM); 200 γ/0.3 ml. of folic acid :final conct. 1.52×10^{-4} *M*); 1000 γ/0.3 ml. of SH-glutathione :final conct. 1.1×10^{-3} *M*); 0.1 ml. of 10^{-2} *M* of $CoCl_2$ and of $CdSO_4$ (final conct. 3.3×10^{-4} *M*).

of rabbit by the Procedure II (dialized enzyme B) of Kato *et al.* and confirmed, that this enzyme preparation was also reactivated by the addition of SH-glutathione, folic acid and Co^{++} but Cd^{++} was effectless. As Kato *et al.* already suggested, it became clear, that the disaccordance between two laboratories based on the difference of the experimental animals. Table VII is the data obtained in co-operation by Kato and Koizumi.

SUMMARY

1. The effect of folic acid and SH-glutathione on histidine deaminase of the rabbit, which was reported in the previous papers, was reconfirmed (*1*). In this report Co^{++} was found to be another essential supplement.

2. When EDTA was added in the process of enzyme purification, the activity of the purified enzyme was no longer restored by Co^{++}, Mn^{++}, Zn^{++} or Cd^{++}, but with addition of folic acid, SH-glutathione and Co^{++}, the inactive enzyme regained its activity. The activation of Mn^{++}, Zn^{++} and Cd^{++} was discussed.

3. From one molecule histidine, one molecule ammonia was produced by the thus activated enzyme and as the metabolite urocanic acid was proved by means of paperchromatography.

4. Folinic acid was found not to have such a restoring effect on histidine deaminase.

5. The disaccordance between Kato *et al.* (*12*) and the authors was discussed and it became clear, that this was to be attributed to the difference of the experimental animals.

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STUDIES ON PROTEIN DENATURATION BY SURFACE CHEMICAL METHOD

IV. ON THE STRUCTURE OF LYSOZYME MONOLAYER

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In the previous papers (*1–3*), the author discussed the relationship of the monolayer properties of lysozyme to its denaturation in bulk. This paper is concerned with a detailed examination of the structure of lysozyme monolayer by the measurements of surface viscosity and surface potential in addition to surface pressure.

An account has been given of the kinetics of surface denaturation of lysozyme in Part II, where some assumptions on the structure of lysozyme monolayers were inevitably necessary (*2*). These assumptions have been substantiated by the present surface potential measurements.

As has been described in Part I, five intrapeptide disulfide bridges existing in a small lysozyme molecule might be essential for its resistance against denaturants (*1*). The change in the reactivity of lysozyme molecules caused by splitting of these disulfide bridges are interesting for monolayer studies.

It has been clarified recently that intermolecular disulfide bonds are formed in the course of denaturation and that these bridges affect profoundly the various physicochemical properties of denatured proteins (*4–8*). The possibility that the similar effect might be also observed for protein monolayers should be examined in detail. From this point of view the monolayer of ovalbumin was examined by the surface viscosity measurement.

EXPERIMENTAL

Crystalline lysozyme and ovalbumin used in this experiment were kindly supplied by Prof. S. Akabori, Mr. K. Ohno and Mr. I. Haruna in the Faculty of Science of Osaka University.

The surface pressure-area curves of lysozyme monolayers were changed by the

spreading solutions as reported in Part I and II. Following solutions were, therefore, used for spreading: (a) the solution in which lysozyme was dissolved in 8 *M* urea. Before spreading 40 per cent of isopropyl alcohol was added to this solution; (b) the solution in *N*/1000 hydrochloric acid; (c) the solution in *N*/1000 hydrochloric acid to which 2 per cent of isopropyl alcohol was added. Ovalbumin was spread from its solution in 8 *M* urea to which 40 per cent of isopropyl alcohol was added before spreading.

Sodium sulfite was used for reducing the disulfide bonds in lysozyme molecules. 3 ml. of lysozyme in 8 *M* urea solution was pipetted into a test-tube, to which 0.5 ml. of 1 *M* sodium sulfite was added. The test-tube was then immersed in a thermostat at 30° for one and half an hour. By this treatment the solution became positive for nitroprusside test. Then, 30 per cent of isopropyl alcohol was added before spreading.

With lysozyme monolayers aqueous solution of potassium carbonate in concentration of 0.01 *M* (pH 10.5) was used as substrates. In the case of ovalbumin the pH's of the substrates were adjusted by hydrochloric acid for lower pH values and by potassium carbonate for higher pH's. When the disulfide bridges were reduced on the surface, 0.01 *M* of sodium sulfite was added to the substrates.

Surface pressure was measured by the hanging plate method of Wilhelmy type. Simultaneously surface potential was measured by vibrating electrode method. Surface viscosity was measured by the damping of the oscillatory rotation of disc on the surface covered with monolayers (*9*).

RESULTS AND DISCUSSION

Surface Pressure-Area (F-A), Surface Viscosity-Area (η-A) and Surface Potential-Area (ΔV-A) Curves of Lysozyme

The F-A, η-A and Δ V-A curves of lysozyme monolayers spread from 8 *M* urea containing 40 per cent of isopropyl alcohol, from a solution in *N*/1000 hydrochloric acid and from a solution in *N*/1000 hydrochloric acid containing 2 per cent of isopropyl alcohol are shown in Figs. 1, 2 and 3, respectively. The surface moment-area (μ-A) curves are also shown in these figures. The surface moment, μ, was calculated from Helmholtz's formula,

$$\mu = \frac{\Delta V}{4\pi n} \qquad (1),$$

where ΔV is the surface potential and n the number of residues per unit area.

The monolayer properties of lysozyme spread from the solution in 8 *M* urea were examined especially in detail because in this case the monolayer of the protein completely denatured by surface was obtained as reported in Part I and II. The Δ V-A curves were determined ten

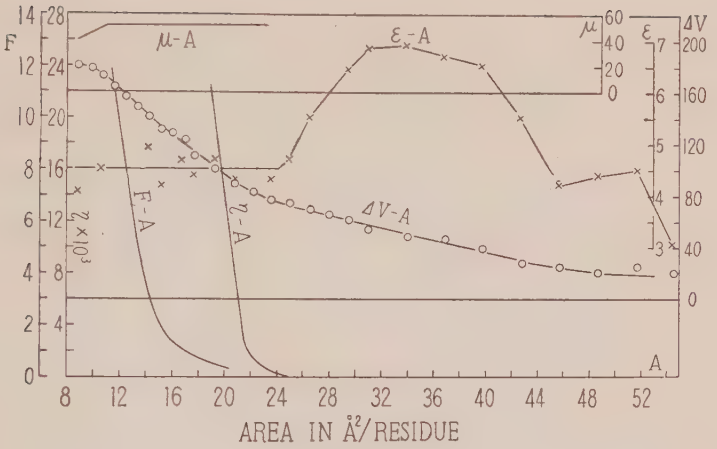

FIG. 1. F-A, η-A, ΔV-A, μ-A and ε-A curves for monolayer of lysozyme spread from 8 M urea solution containing 40 per cent of isopropyl alcohol; substrate, 10^{-2} M potassium carbonate (pH 10.5). 19°. F in dynes/cm., η in surface poises, ΔV in millivolts, μ in millidebyes.

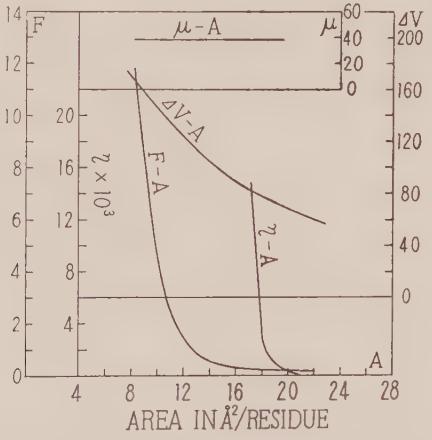

FIG. 2. F-A, η-A, ΔV-A and μ-A curves for monolayer of lysozyme spread from N/1000 hydrochloric acid; substrate, 10^{-2} M potassium carbonate (pH 10.5) 14°.

times under the same condition and their mean values are plotted in

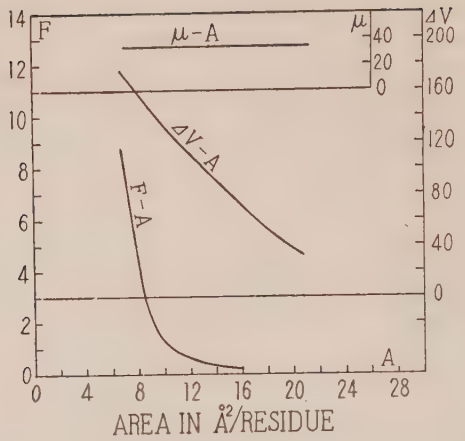

FIG. 3. F-A, ΔV-A and μ-A curves for monolayer of lysozyme spread from $N/1000$ hydrochloric acid containing 2 per cent of isopropyl alcohol; substrate, 10^{-2} M potassium carbonate (pH 10.5). 15°.

Fig. 1. The mean errors of these observed ΔV values are also plotted in Fig. 1 (ε-A curve). The mean deviations were small at higher areas and then became larger as the film was compressed. However, the fluctuation in surface potential became smaller again below the area of about 22 Å²/residue. It is very remarkable that this corresponds to the area from which the surface viscosity increases steeply. It is the reason for the high reproducibility of the surface potential at sufficiently large area that each molecule of lysozyme might be able to behave independently and uniformly on water surface. As the film was compressed, however, the protein molecules might be clustered together into non-uniform "islands" and so the surface potential fluctuates considerably. The similar observation was made by Harrap (*10*) and by Cockbain and Schulman (*11*) for the films of insulin and gliadin, respectively. Though his measurement was not made of surface viscosity, Harrap measured the surface potential of insulin films by ionizing electrode method over a large pH range. He pointed out that the region in which surface potential fluctuated violently appeared especially in the vicinity of its isoelectric point. The lowest net charge on protein molecules at the isoelectric point favors for their aggregation into "islands." In the present experiments as well, the surface potentials of the lysozyme films were measured at its isoelectric point.

When the film was compressed further until the surface viscosity increased rapidly, all the lysozyme molecules in the monolayer approach each other closely and uniformly and so the values of surface potential become reproducible. It is considered, therefore, that the area from which the surface viscosity increases rapidly corresponds to the area in which the protein molecules approach sufficiently closely.

It is interesting to note that the change in the state of the lysozyme monolayer accompanied by compression might be inferred from the ε-A curve. That is, the lysozyme film is in a gaseous state above the areas of about 50 Å^2/residue. Below this area the molecules tend to aggregate into "islands." Further compression of the film below 22 Å^2/residue causes the gelling of the film. A similar behaviour was also found for the results shown in Figs. 2 and 3.

All the F-A curves shown in Figs. 1, 2 and 3 are of condensed type and the surface viscosity is very high at lower surface pressures. It was pointed out previously that this was also the case for the films of amphoteric copolypeptides at their isoelectric points. That is, the films which give the F-A curves of condensed type are in the isoelectric state and are characterized by the high viscosity even at lower surface pressures (*12*, *13*).

The Relationship between Surface Potentials and Surface Pressures of Lysozyme (Δ V-F Curve)

Comparing the results shown in Figs. 1, 2 and 3, it was found that the area of the film decreased in the order: the film spread from the 8 *M* urea solution containing 40 per cent of isopropyl alcohol, from *N*/1000 hydrochloric acid and finally from *N*/1000 hydrochloric acid containing 2 per cent of isopropyl alcohol.

In Part II, where the mechanism of surface denaturation of lysozyme was examined, the results were analyzed on the following two assumptions: (a) The maximum expansion obtained for the film spread from 8 *M* urea solution corresponds to the area of the monolayers of lysozyme denatured completely by surface, and the films having smaller areas than this maximum expansion consists of two different type of molecular configurations, one of which is in a state of complete surface denaturation and the other, globular or partially altered configuration; (b) The latter configuration was assumed by the molecules existed in the adsorbed layer and does not contribute to surface pressures. By these assumptions the concentration of the "globular" molecules (C_n) was represented by the equation:

$$\frac{1}{A} - \frac{1}{A_d} = C_n \qquad (2),$$

Where A_d is the area of maximum expansion and A the area at any given expansion.

These assumptions were described by Cheesman and Schuller in their paper on the surface inactivation of pepsin (*14*). Though they did not publish the detailed results, their assumptions were based on the fact that the film potential of difficultly spread proteins such as myosin was found to be characteristic of the pressure irrespective of the degree of spreading.

The ΔV-F curves constructed from the curves in Figs. 1, 2 and 3 are shown in Fig. 4.

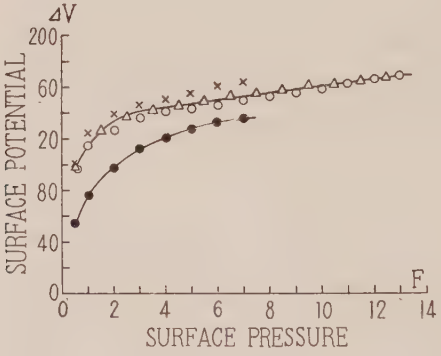

FIG. 4. ΔV-F curves for lysozyme monolayers spread from 8 *M* urea containing 40 per cent of isopropyl alcohol (△), from *N*/1000 hydrochloric acid (○) and from *N*/1000 hydrochloric acid containing 2 per cent of isopropyl alcohol (×). Substrate, 10^{-2} *M* K_2CO_3. (●), spread from 8 *M* urea containing 40 per cent of isopropyl alcohol. Substrate, 10^{-2} *M* $K_2CO_3 + 10^{-2}$ *M* Na_2SO_3.

It is found from Fig. 4 that in the case of lysozyme as well, the relations between the film potentials and the surface pressures are represented by the same single curve, irrespective of the spreading solutions.

It was reported in Part II that the F-A curves were shifted to larger areas with the age after spreading and that the relationship between the logarithms of C_n and time was expressed by a straight line. This is the indication for the first order process of surface denaturation of " globular "

molecules.

These two facts substantiated the assumptions that the monolayers of lysozyme consist of the molecules in a state of complete surface denaturation and of the molecules having globular or partially altered configuration existed in adsorbed layer. Therefore, the procedure described in Part II should be correct.

The expansion of the lysozyme film spread from its solution in $N/1000$ hydrochloric acid containing 2 per cent of isopropyl alcohol was smaller than that of the film spread from the solution without alcohol. Isopropyl alcohol may be likened to a retarding agent against surface denaturation of lysozyme. Further studies are necessary on the effect of alcohol on the protein.

Effect of Sodium Sulfite on the Monolayers of Lysozyme and Ovalbumin

In Fig. 5 are given the F-A, η-A, Δ V-A and μ-A curves of lysozyme monolayers spread from 8 M urea solution on the substrate containing 10^{-2} M sodium sulfite in addition to potassium carbonate (pH 11).

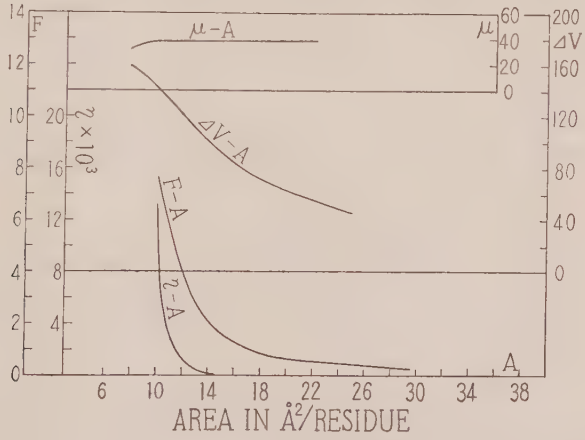

FIG. 5. F-A, η-A, Δ V-A and μ-A curves for lysozyme monolayer spread from 8 M urea solution containing 40 per cent of isopropyl alcohol. Substrate, 10^{-2} M K_2CO_3+10^{-2} M Na_2SO_3.

Following five distinct differences are to be noted between the results in Fig. 5 and those in Fig. 1 which were obtained for the mono-

layers on the substrate without sodium sulfite.

(a) The F-A curve was of expanded type and the η-A curve developed at the area of about 15 Å^2/residue when sodium sulfite was present in the substrate. However, the F-A curve was of condensed type and the η-A curve had the limiting area of about 22 Å^2/residue on the substrate without sodium sulfite.

(b) The film on the substrate with sulfite was not sensitive for surface viscosity until the film pressures were rather high. Without sulfite, on the contrary, the surface viscosities were already high at lower surface pressures.

(c) In the presence of sulfite, reproducibility of surface potentials was very high from larger areas. In the absence of sulfite, as described above, there was a correspondence between the area from which the surface viscosities increased rapidly and that from which the values of surface potentials became reproducible. Such a correspondence was not observed for the film on the substrate containing sulfite.

(d) Comparing the Δ V-A curve in Fig. 1 the values of surface potential were as low as 50 mV. in the presence of sulfite.

(e) The Δ V-F curves shown in Fig. 4 were different from each other by whether sodium sulfite was present or not.

The presence of 10^{-2} *M* sodium cyanide in the substrate also changed the F-A curve to an expanded type. Sodium sulfate, however, had no effect on the F-A curve.

Sodium sulfite or cyanide are known to be the splitting agent for disulfide bonds. In the former case the splitting is thought to proceed by the following reaction (*7*).

$$RSSR + SO_3^{--} \rightleftarrows RS^- + RSSO_3^-.$$

When five disulfide bridges internally crosslinked in lysozyme molecules are completely reduced by sulfite, negative charges were increased by ten per molecule. The isoelectric point of the reduced lysozyme, therefore, no longer lies near pH 11.

Previously, the author examined the properties of the monolayers of amphoteric copolypeptides of lysine, leucine (or phenylalanine) and glutamic acid over a wide pH range (*12*, *13*). It was found that while the F-A curve at the isoelectric point was of condensed type and the surface viscosities were already high at lower pressures, in both the outside of the isoelectric point the F-A curves were changed to an expanded type and the viscosities were not large until the surface pressures became considerably high. These facts could be interpreted in terms of free

net charge and salt linkage. In the present experiments the situation is quite similar, that is, the reason for the facts described in (a), (b) and (c) must be ascribed to the increase in net negative charge, resulting from the reduction of the disulfide bonds. Because of the intermolecular repulsion due to the increase in the negative charge, the film was not sensitive for surface viscosities above the area of 15 Å²/residue which is characteristic to a peptide bond in backbone (*15*). The high reproducibility of surface potentials over a wide range of area might be caused by the electrostatic repulsion preventing the formation of "islands."

Below are described the monolayer properties of lysozyme on the substrate with or without sodium sulfite when the film is spread from a solution in which the protein has been reduced by sulfite beforehand.

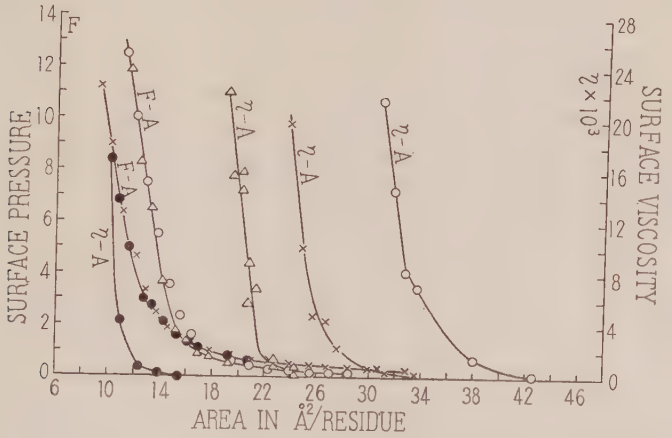

FIG. 6. F-A and η-A curves for lysozyme monolayers spread from 8*M* urea solution in which lysozyme is treated with sodium sulfite (○, ×) and from 8 *M* urea solution of untreated lysozyme (△, ●). Substrates, 10^{-2} *M* K_2CO_3 (○, △), 10^{-2} *M* $K_2CO_3+Na_2SO_3$ (●, ×).

Fig. 6 shows the effect of sodium sulfite in the substrate on the F-A and η-A curves for the monolayers of lysozyme which was treated with sulfite in 8 *M* urea solution prior to spreading. The F-A and η-A curves for the untreated lysozyme on the substrates with or without sulfite are also shown for comparison in this figure. While the F-A curves of lysozyme reduced in its spreading solution beforehand resembled those of the untreated protein, the η-A curves were very distinctly different from

each other. It is noteworthy that it would commit an error to discuss the properties of protein monolayers only from their F-A curves and that surface viscosity measurements prove to be of great value in investigating protein monolayers.

The η-A curve for the films of the treated protein on the substrate without sulfite developed from very large area. When sodium sulfite was present in the substrate, however, the limiting area of the η-A curve became smaller. This fact shows that in the former case some but not all of the intermolecular bridges are made of disulfide bonds and that these bridges are response for the very large viscosities. The viscosity is decreased by the presence of sulfite due to the reduction of these intermolecular disulfide bridges. In the presence of sulfite in the substrate, however, the η-A curve for the treated lysozyme was not decreased to the same limiting area (about 15 $Å^2$/residue) as the curve for the untreated protein. This is the indication for the presence of some kinds of strong intermolecular bridges in addition to disulfide bonds. The question arises as to the origin of these disulfide bridges: whether the latter bonds are formed by the oxidation of the mercaptide ions owing to the absence of sulfite in the substrate, or the addition of 30 per cent of isopropyl alcohol, which is necessary for spreading, causes the intermolecular cross links including disulfide bonds. Fraenkel-Conrat, Mohammad, Ducay and Mecham (*16*) have found that the dimerization of lysozyme molecule reduced by thioglycol occurred when the reduced protein was precipitated with acetone or ethanol. They considered that the dimerization might be due to the formation of a disulfide bond between single particularly reactive sulfhydryl group on pairs of reduced lysozyme molecules. It is generally observed that protein molecules are aggregated or precipitated by the reactivity of otherwise inaccessible groups liberated by splitting of disulfide bonds. It is, therefore, more appropriate to consider that the strong aggregates had been already formed in the spreading solution rather than that mercaptide ions are oxidized to form the disulfide bonds only after spreading.

It was found from these experiments that the reactivity of reduced lysozyme is very different from that of the untreated protien. Furthermore, the present experiment shows that the presence of intermolecular disulfide bonds contribute considerably to the increase in surface viscosity. Previously we have pointed out that a correlation exists between surface viscosity and the number of salt linkages in the monolayers of

amphoteric copolypeptides (*12*, *13*). It is to be born in mind that in protein films disulfide bonds in addition to salt linkeages contribute to the increase in surface viscosities.

It has become clear recently that intermolecular disulfide bridges produced by the exchange reaction between the sulfhydryl and disulfide groups of denatured proteins affect the various physico-chemical properties of protein solutions, such as viscosity, turbidity or sedimentation (*4-8*). It is necessary to prove the existence of such bridges in protein monolayers and to examine the effect of these bonds, if present, on the properties of protein monolayers. Expecting the greater contribution of intermolecular disulfide bonds to surface viscosity, the change in surface viscosities of ovalbumin monolayers by the presence of sodium sulfite in substrates was examined, with the results shown in Fig. 7. Ovalbumin contains only one disulfide and five sulfhydryl groups per molecule.

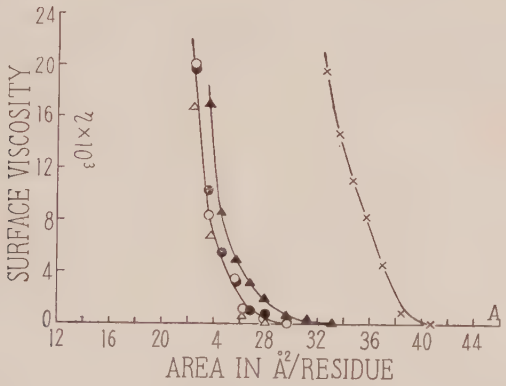

FIG. 7. η-A curves for ovalbumin monolayers spread from 8 *M* urea solution containing 40 per cent of isopropyl alcohol. Substrates, pH 4.6 (HCl) (×). pH 7.6 ($KHCO_3$) (◒), pH 10.5 (K_2CO_3) (●), pH 8.2 ($KHCO_2+NaSO_3$) (△), pH 11 ($K_2CO_3+Na_2SO_3$) (○).

Such a pronounced effect as in the case of lysozyme was not found for the ovalbumin monolayers and the evidence of the existence of such inter- or intra-molecular disulfide bridges could not be detected. The surface viscosity was maximum at its isoelectric point and the relation between the surface viscosities and the pH's of substrates resembled that for amphoteric polypeptides (*12*, *13*). Frensdorf, Watson and

Kauzmann (7) have pointed out that the optimum for the gelation of ovalbumin in 10 *M* urea solution was considerably higher than the isoelectric point for urea-denatured ovalbumin (pH 5.7), at pH 9. The correspondence for their results was not observed in the case of ovalbumin monolayers.

SUMMARY

The structure of lysozyme monolayers was investigated by the measurements of surface pressure, surface viscosity and surface potential. Following four distinct results were obtained.

(1) The reproducibility of the surface potential-area curve of lysozyme was very high at larger areas. The region in which film potentials fluctuate violently appeared as the film was compressed. Further compression beyond the area from which the surface viscosities began to rise steeply made the reproducibilities of the potentials very high. These facts are related to the aggregation state of lysozyme molecules. Therefore, the change in the state of lysozyme monolayer at its isoelectric point might be inferred from the curve of mean deviations of the observed ΔV values plotted against areas.

(2) The curves of surface potentials plotted against pressures were represented by a single curve and did not depend on the spreading solutions. The assumptions for the structure of lysozyme monolayers described in Part II were substantiated from this fact.

(3) The presence of sodium sulfite affects the F-A, η-A and ΔV-A curves of lysozyme monolayer. The reduction of intramolecular disulfide bonds were shown from various observations. When lysozyme was reduced with sulfite in spreading solution, the monolayers spread from it had very high viscosities from larger areas. The formation of strong intermolecular crosslinks including disulfide was suggested in this case.

(4) The presence of sodium sulfite in the substrate had no effect on the viscosities of ovalbumin monolayers. In this case the monolayers were spread from a solution of ovalbumin in 8 *M* urea. Nevertheless, the evidence of the formation of inter- or intra-molecular disulfide bonds was not obtained.

The author wishes to express his sincere thanks to Prof. T. Isemura for his kind guidance throughout the present work and to Prof. S. Akabori, Mr. K. Ohno and Mr. I. Haruna in the Faculty of Science of Osaka University who supplied the valuable samples of lysozyme and ovalbumin.

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FORMATION OF BICONCAVE DISCOID FORM OF OIL DROPLET IN AQUEOUS PROTEIN SOLUTION

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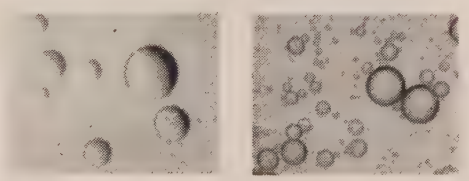
(Received for publication, March 2, 1956)

As is well known, normal mammalian red blood cell has a biconcave circular disk form. There seems to exist no plausible explanation why and how such a form comes to appear. Since cell content is liquid and not solid with any structure, it should be caused by some chemical or physical forces attached to the enclosing membrane, namely stroma. Many trials have been done with the expectation that any emulsoid particle can be transformed experimentally to biconcave circular disk form. Fortunately the authors met with success to bring about this form on the emulsoid of fatty acid or oil in the protein solution. Caprylic acid and once boiled sesame oil, for example, assume usually spherical form when dispersed in aqueous saline solution as shown in Fig. 1. These emulsions are of course unstable unless a stabiliser is present. When we add egg white as such stabiliser, we can get highly stable emulsoid, the particles of which are enclosed with creasy protein membrane (Fig. 2). At the certain conditions with regards to the concentrations of

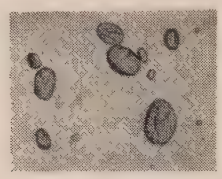


Sesame oil (smooth sphere) Caprylic acid (smooth sphere)

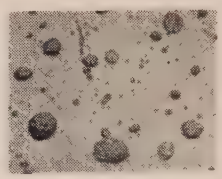
Fig. 1. Droplet in saline solution.

protein, salt and pH, they turn out to hold biconcave circular disk form as shown in Fig. 3. And the degree of " biconcavity " increase gradually to reach doughnut form (Fig. 4). This finding is quite interesting, for it seems to afford a clue to the elucidation of this peculiar form of ery-

throcyte. In this report no mention is made on the essential mechanism involved in the transformation, but simply the details of the experiment led to this finding will be given.



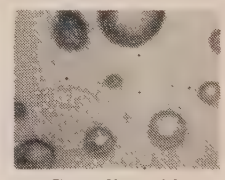
Sesame oil (creasy oval)



Caprylic acid (creasy oval)

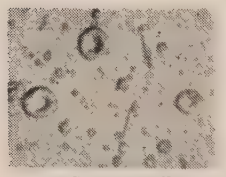
FIG. 2. Creases on the surface of droplet in protein solution.

Sesame oil (creasy biconcave disk)

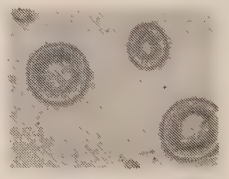


Caprylic acid (creasy biconcave disk)

FIG. 3. Begining of biconcavity.



Sesame oil (creasy biconcave disk)



Caprylic acid (creasy biconcave disk)

FIG. 4. Advanced biconcavity.

EXPERIMENTAL

Materials—Egg white of the white Leghorn used throughout the experiments was separated as completely as possible from the yolk and filtered with a piece of gauze. As dispersion materials, caprylic acid and once boiled sesame oil was used. Physical and chemical properties of this sesame oil heated at the temperature 220–250° for one hour were as follow (Table I).

Observation of the Shape of Dispersed Droplet—Suitable amounts of saline solution were placed in test tubes and then caprylic acid or sesame oil was added to. After vigorous

TABLE I

Physical and Chemical Properties of once boiled Sesame Oil
(boiled at 220–250° for 1 hour)

Material	Relative viscosity (30°) (Ostwald viscosimeter)	Specific gravity (30°) (Sprengel pycnometer)	Peroxide value (Wheeler, *mg*/100 *g*)	Iodine value (Wijs)	Acid value
Raw	33.0	0.919	3.5	107	1.0
Boiled	54.8	0.929	13.0	104	1.2

shaking, egg white solution was added to each tube. Dispersed droplets which are 20–50 μ in diameter were observed under microscope at definite intervals of time.

Determination of the Protein bound to the Interface—This was done following the method of Przylecki *et al.* (*1*). Caprylic acid was well dispersed in saline solution containing egg white. After standing 5 minutes, this emulsion was centrifuged and the upper caprylic acid layer was discarded. The rest of the solution was filtered through the Toyo filter paper No. 50. Similar treatment with sesame oil was carried out. In this case the emulsion was centrifuged after standing one hour. The protein contents in these filtrates were measured by means of the biuret reaction (*2*) spectrophotometrically and compared with the original protein content. The diminution of protein concentration in the dispersion medium is assumed to be a bound amount.

RESULTS

1) The Effect of the Egg White—The shapes of caprylic acid droplets of 20–50 μ in diameter dispersed in 0.3 mol saline solutions, in which the concentration of egg white was variable, were observed under microscope at the end of 5 minutes after dispersion. The results are shown in Table II. It is apparent that the more increased concentration of egg white seems to cause the more rapid and the more noticeable formation of the creases on the surface of droplets. Further, it is seen that droplets are biconcave disk in the solution of over 0.1 per cent of protein concentration, while they are oval in 0.05 per cent and spherical below 0.01 per cent. But 20 minutes later the shape of droplets was transformed to biconcave disk even in the solution of 0.05 per cent.

The effect of increasing amount of protein on the shape of sesame oil is seen in Table III. In this case, the transformation of droplets appeared more slowly than in the case of caprylic acid, and biconcave discoid form were obtained in the protein solution of over 4.9 per cent

TABLE II

Influence of Protein Concentration on the Shape of Caprylic Acid Droplets

(saline concentration 0.3 *M*, pH 5.2)

Concn. of protein	Shape of droplet
%	
0.01	Smooth sphere
0.05	Creasy oval
0.1	Creasy biconcave disk
0.5	"
1.0	"
2.4	"
4.9	"

TABLE III

Influence of Protein Concentration on the Shape of Sesame Oil Droplets

(saline concentration 0.3 *M*, pH 9.2)

Concn. of protein	Shape of droplet
%	
0.5	Smooth sphere
1.0	"
1.5	Creasy sphere
2.0	Creasy oval
2.4	Creasy disk
4.9	Creasy biconcave disk
6.5	"

after one hour. Moreover, it was noticed that in the case of sesame oil the transformation appeared at first on larger droplet, while in the case of caprylic acid on smaller one.

2) *The Effect of Saline Concentration*—From the above results it was decided to use protein concentration over about 2.4 per cent for studying the effect of saline concentration. Two drops of caprylic acid or 4 drops of sesame oil were mixed and stirred with 1.0 ml. of saline solutions of various concentrations in test tubes, and then 1.0 ml. of

the egg white solution containing NaOH was added to each tube. Thus, the end concentration of salt as indicated in Tables IV and V were obtained. Both pH and the protein concentration of the whole solution were constant as shown in tables.

The shape of caprylic acid droplets was observed after standing for 5 minutes, and that of sesame oil droplets after one hour. As can be seen from Tables IV and V, the transformation of droplets was more

TABLE IV

Influence of Saline Concentration on the Shape of Caprylic Acid Droplets

(protein concentration 2.3 per cent, pH 5.6)

Concn. of NaCl	Shape of droplet	Amount of protein* coagulated around droplet
M		*g.*
0.05	Creasy biconcave disk	0.032
0.1	"	0.046
0.2	"	0.043
0.3	"	0.045
0.4	"	0.040
0.5	Creasy oval	0.015
0.7	"	0.011
1.0	"	0.006
1.5	Creasy sphere	0.005

* Means the decrease of protein in 10 cc of 2.3 per cent protein solution.

remarkable in lower concentration of salt. Namely, in the range of 0.1–0.3 *M* (sesame oil) and 0.05–0.4 *M* (caprylic acid) droplets showed biconcave discoid form. When, however, pure water was used instead of saline solution the shape of oil droplets was fibrous and creases were scanty.

In the last column of tables, amount of protein bound to the surface of droplets are shown. It is clear that the transformation was proportional to the amount of this bound protein.

3) The Effect of pH—Emulsion was similarly prepared as above. Egg white was added to the mixture of saline solution and caprylic acid or sesame oil. These solutions in which saline concentration was constant (0.3 *M*) were brought to various pH as shown in Tables VI and

VII by adding HCl or NaOH solution. pH was determined with a glass electrode pH-meter. The results are given in Tables VI and VII. The shape of caprylic acid droplets was biconcave disk in the range of

TABLE V

Influence of Saline Concentration on the Shape of Sesame Oil Droplets

(protein concentration 4.9 per cent, pH 8.8)

Concn. of NaCl	Shape of droplet	Amount of protein* coagulated around droplet
0 *mol*	Fibrous	—
0.1	Creasy biconcave disk	0.018 *g*
0.2	"	0.015
0.3	"	0.015
0.4	Creasy oval	0.010
0.5	"	0.008
0.7	"	0.007
1.0	Creasy sphere	0.001
1.5	Smooth sphere	0.001

* Means the decrease of protein in 10 cc of 2.45 per cent protein solution

TABLE VI

Influence of pH on the Shape of Caprylic Acid Droplets

(saline concentration 0.3 *M*, protein concentration 2.3 per cent)

pH	Shape of droplet	Amount of protein* coagulated around droplet
1.8	Creasy sphere	— *g.*
3.5	Creasy oval	0.017
4.8	"	0.020
5.6	Creasy biconcave disk	0.038
6.2	"	0.044
6.8	Creasy oval	0.025
7.1	"	0.017

* Same as Table IV.

TABLE VII

Influence of pH on the Shape of Sesame Oil Droplets
(saline concentration 0.3 *M*, protein concentration 4.9 per cent)

pH	Shape of droplet	Amount of protein* coagulated around droplet
		g.
5.5	Smooth sphere	0.001
6.9	"	0.003
7.4	Creasy oval	0.008
8.2	Creasy disk	0.020
9.4	Creasy biconcave disk	0.020
10.1	Creasy oval	0.014
11.2	Creasy sphere	0.009

* Same as Table V.

pH 5.6–6.2 (optimum pH), and at the acid side than pH 5.6 or at the alkaline side than pH 6.2 the creases on the surface were diminished and the degree of the transformation of droplets became more slight. However, the optimum pH in the case of sesame oil was about 8.2–9.4. As seen in the last column of tables, the transformation was proportional to the amount of protein bound to the surface of droplets.

4) *The Effect of Temperature*—Emulsions of sesame oil were prepared in the solution containing 0.3 *M* salt and 4.6 per cent protein and adjusted at pH 8.2. As temperature 2°, 10°, 20°, 30°, 40°, and 50° were chosen to avoid the heat denaturation of protein. As shown in Table VIII, the transformation was more remarkable in lower temperature.

TABLE VIII

Influence of Temperature on the Shape of Sesame Oil
(saline concentration 0.3 *M*, protein concentration 4.6 per cent)

Temperature (C)	Shape of droplet
2°	Creasy biconcave disk
10°	"
20°	"
30°	Creasy oval
40°	Creasy sphere
50°	Smooth sphere

In the case of caprylic acid, however, the effect of temperature was not so clear in the range of 20–50°.

SUMMARY

1. The shape of caprylic acid or once boiled sesame oil in a saline solution is spherical. When egg white is added, the shape is transformed from sphere to disk and at the same time the creases appear on the surface of droplets.

2. Under suitable conditions of the dispersion medium with regard to NaCl concentration and pH, it turns out to be biconcave discoid. In the case of caprylic acid such salt concentration exists in the range of 0.05–0.4 *M* and pH of 5.6–6.2, and in the case of sesame oil the salt concentration in the range of 0.1–0.3 *M* and pH at 8.2 –9.4.

3. These phenomena are related to the coagulation of protein on the oil-water interfaces.

We wish to thank heartly to Dr. Keizo Kodama, President of Tokushima University, for his kind revision.

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AEROBIC OXIDATION OF TRIOSE REDUCTONE BY CRYSTALLINE TURNIP PEROXIDASE

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(Received for publication March 2, 1900)

It is well known that horse-radish peroxidase acts as an aerobic oxidase in the presence of dihydroxymaleic acid (DHM) and that Mn^{++} is the most effective activator for this reaction. Chance (*1*) investigated the reaction by his special instruments and confirmed the successive reactions as shown below:

$$O_2 + DHM \xrightarrow[Mn^{++}]{HRP} H_2O_2 + DKA^*$$

$$H_2O_2 + DHM \xrightarrow{HRP} 2\ H_2O + DKA$$

Kenten and Mann (*2–4*) showed that several other substances were also oxidized by the peroxidase system under their experimental conditions.

In our earlier experiments, it was found that the aerobic oxidation of triose reductone (RnH_2) was catalyzed by the oxidase distributed in many plant tissues, especially in roots. Later, this RnH_2-oxidase was found to be identical with a peroxidase in our laboratory. The results of kinetic investigation of the RnH_2-oxidase reaction resemble those which have been obtained by Chance, but, unlike DHM and other substrates ever known, RnH_2 is markedly oxidized by a low concentration of crystalline peroxidase in the absence of Mn^{++}. In the aerobic oxidation of RnH_2 by peroxidase, Mn^{++} does not play an essential role, but the trace amount of H_2O_2 is indispensable to the reaction.

EXPERIMENTAL

RnH_2 was prepared by the method of Euler (*5*) and recrystallization was repeated three times. The turnip peroxidase was purified by the slightly modified method

* DKA: Diketo acid of DHM.

of Kondo and Morita (*6*), and its crystallization was carried out by Kenten and Mann's method (*7*). Recrystallized turnip peroxidase was used all through the experiments. Peroxidase concentration was calculated by assuming that the extinction coefficient of turnip peroxidase is 90 cm.$^{-1}$mM^{-1} at 403 mμ.

The reactions were stopped by the addition of 1 ml. 3 N-H_2SO_4 to 5 ml. reaction mixture and RnH_2 remained was determined by titration with 1 mM iodine solution. Thereafter, 1 ml. per cent-KI and 1 drop of saturated ammonium molybdate were added to each solution and the amount of H_2O_2 was also determined by the iodometric method. Optical density was determined by the Beckmann spectrophotomerter, DU type.

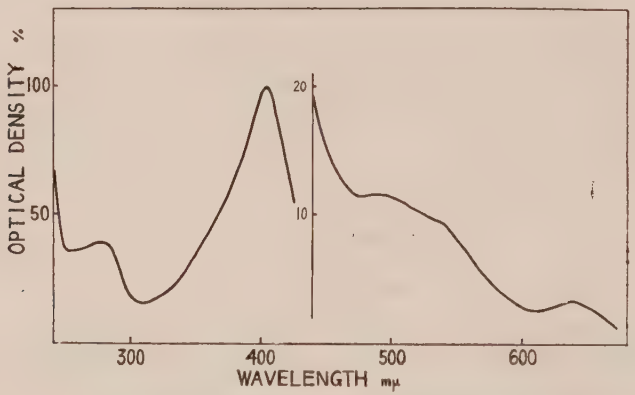

Fig. 1. Absorption spectrum of crystalline turnip peroxidase. Optical density in the visible band is in 5 times large scale as that in the ultraviolet and Soret bands.

RESULTS

The rate of RnH_2 oxidation depends upon the peroxidase concentration but no proportionality is obtained between the oxidation rate and the peroxidase concentration, as shown in Fig. 2. In the low concentration of peroxidase, H_2O_2 accumulates during the reaction process and steady state reaction appears after a short lag phase period. If appropriate amount of H_2O_2 is added to the reaction mixture, steady state rate is obtained from the beginning of the reaction (Fig. 3). With increasing peroxidase concentration, the accumulation of H_2O_2 during the reaction process decreases. At the peroxidase concentration above 0.1 μM no H_2O_2 is detectable in the reaction mixture and the reaction rate becomes almost constant. In our experimental conditions, maximal

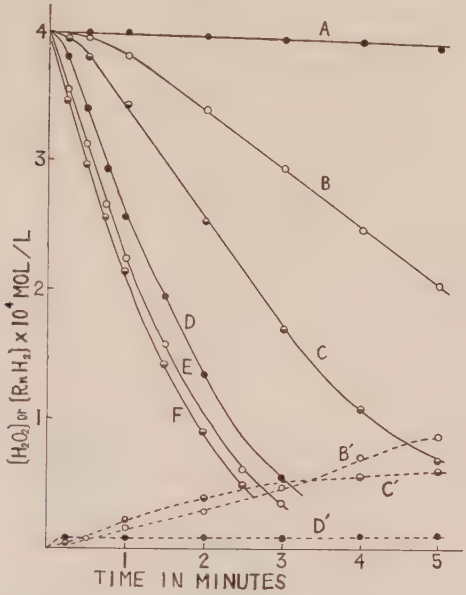

FIG. 2. Reaction curves of RnH_2 oxidation in 0.01 *M* acetate in the presence of various amounts of peroxidase.

pH 5.3: temperature 25°. Solid lines show decrease of RnH_2 and broken lines show amounts of H_2O_2 produced during the reaction process.

Concentrations of peroxidase are as follows: A, 0; B and B′ 0.0008 μM; C and C′ 0.004 μM; D and D′ 0.02 μM; E 0.1 μM; F 0.5 μM. In the cases of E and F no H_2O_2 was detectable.

reaction rate was obtained at pH 5.0–5.5 (Fig. 5) and each reaction curve at various pH is shown in Fig. 3. Fig. 4 shows that 0.1 μM crystalline beef liver catalase or 200 μM ascorbic acid strongly inhibits the reaction. When the reaction mixture is bubbled with O_2 gas instead of the air, the reaction rate increases greatly and a large amount of H_2O_2 is accumulated even in a high peroxidase concentration (Fig. 4).

Table I shows the effect of various metal ions on the oxidase reaction. In this case Mn^{++} is the most effective activator as reported in the DHM-peroxidase system, but the extent of Mn^{++} activation is very small in the RnH_2-peroxidase system as compared with that in the DHM-peroxidase system. In the latter system, Chance (*2*) reported about 20 times

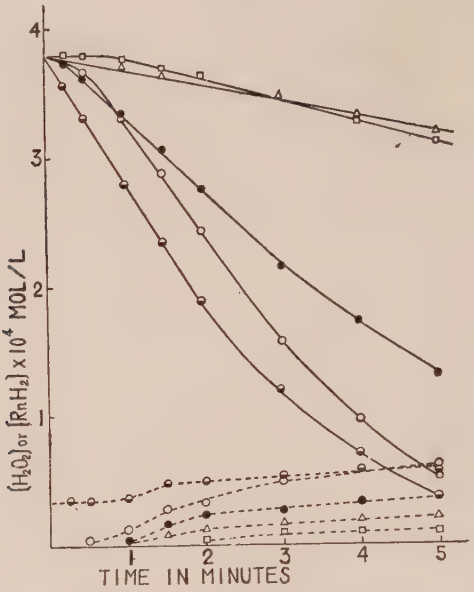

FIG. 3. Effect of pH and H_2O_2 on the RnH_2 oxidation in the presence of 0.004 μM peroxidase at 25°.

Solid lines show decrease of RnH_2 and broken lines show increase of H_2O_2. △: pH 7.3 (0.01 M phosphate). □: pH 4.0 (0.01 M acetate). ●: pH 6.0 (0.01 M acetate). ○: pH 5.3 (0.01 M acetate). ◒: pH 5.3 (0.01 M acetate) +35 μM H_2O_2.

activation of the reaction rate with 50 μM Mn^{++} under his experimental conditions. The same extent of activation is also shown in Fig. 6, but in the RnH_2-peroxidase system, only about 1.5 times activation is obtained with 40 μM Mn^{++} in the optimal pH range (Fig. 5). On the other hand, Cu^{++} exhibits a very interesting effect on the oxidase reaction in Table I and Fig. 5. In the presence of a very small amount of Cu^{++}, the oxidase reaction is inhibited in acidic pH and some activation is obtained in neutral pH. Then the decrease of the H_2O_2 accumulation is observed in the former and the increase in the latter. Cu^{++} activation of the reaction in neutral pH can be applied to the micro-determination of Cu^{++}, as shown in Fig. 6. This has ten times higher sensitivity as those reported previously (*8*, *9*). By this method the Cu^{++} content of 0.01 μM order in the reaction mixture can be determined.

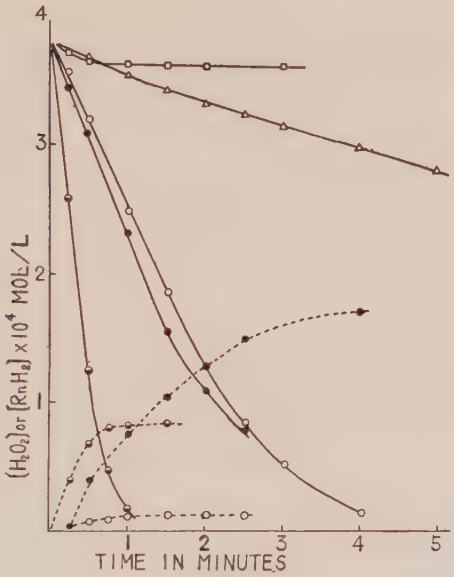

FIG. 4. Effects of catalase, ascorbic acid and O_2 on the reaction curve of RnH_2 oxidation in 0.01 *M* acetate in the presence of 0.02 μM peroxidase (except ●). pH 5.3; temperature 25°. Solid lines show decrease of RnH_2 and broken lines show increase of H_2O_2. □: 0.1 μM crystalline beef liver catalase; △: 0.2 m*M* ascorbic acid (Remaining RnH_2 is obtained from the difference of total reducing amount and added ascorbic acid). ○: control. ◒: O_2 bubbling. ●: O_2 bubbling, 0.0008 μM peroxidase. In the cases of □ and △, no H_2O_2 is detectable.

In the DHM-peroxidase system, Cu^{++} inhibition in acidic pH is also observed as shown in Fig. 7 but no appreciable Cu^{++} activation is obtained in neutral pH.

Hg^{++} itself has no effect on the oxidase reaction in acidic pH. However, curiously enough, Hg^{++} exhibits an inhibitory effect on the oxidase reaction in the presence of a trace amount of Cu^{++} (0.02–0.04 μM). These metal inhibitions are removed completely by the addition of EDTA.

DISSCUSIONS

By the Warburg's manometric experiment the stoichiometry of

TABLE I

Effects of Metal Ions on the RnH_2 Oxidation in the Presence of Peroxidase

No effect*	Inhibition**	Activation***
In acidic range (pH 4–6)		
Hg^{++} (10 μM)	Cu^{++} (0.08 μM)	Mn^{++} (10 μM)
Fe^{++} (,,)	Fe^{++} (10 μM)	
Ag^{+} (,,)		
Zn^{++} (0.4 mM)		
Co^{++} (,,)		
Ba^{++} (,,)		
Ca^{++} (,,)		
In neutral range (pH 7–8)		
Fe^{++} (10 μM)		Mn^{++} (10 μM)
Fe^{+++} (,,)		Cu^{++} (0.08 μM)
Ag^{+} (,,)		Co^{++} (10 μM)
Zn^{++} (0.4 mM)		Hg^{++}: a slight amount
Ba^{++} (,,)		
Ca^{++} (,,)		
Mg^{++} (,,)		

* No effect was observed in the parenthesized concentration.

** Half inhibition in the parenthesized concentration.

*** Half activation in the parenthesized concentration.

the reaction was confirmed as shown in the following equation:

$$RnH_2 + O_2 \rightarrow Rn^* + H_2O$$

Neither H_2O_2 accumulation nor CO_2 evolution was observed under the experimental conditions: pH 4.6, 0.1 μM peroxidase, and 40 μM Mn^{++} or Mn^{++} free. Although this aerobic oxidation seems apparently to be a single oxidase reaction, it is composed of two successive reactions, oxidation and peroxidation, as already reported by Chance in the DHM-peroxidase system.

$$RnH_2 + O_2 \xrightarrow[H_2O_2]{\text{Peroxidase}} Rn + H_2O_2 \qquad (1)$$

$$RnH_2 + H_2O \xrightarrow{\text{Peroxidase}} Rn + 2\ H_2O \qquad (2)$$

The requirement of H_2O_2 in the oxidative Reaction (1) is shown

* Rn: 2 equivalents oxidized form of RnH_2.

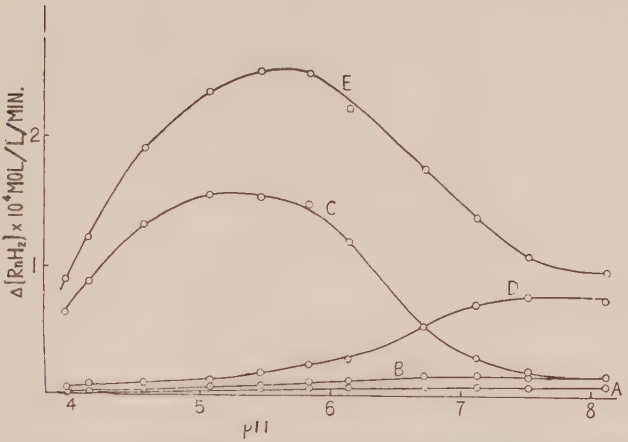

FIG. 5. pH dependence on the initial rate of RnH_2 oxidation under various conditions.

0.4 m*M* RnH_2, temperature 25°. 0.01 *M* acetate for pH 3.95–6.12, 0.01 *M* phosphate for pH 6.70–8.10. A: autoxidation. B: 2 μM Cu++. C: 0.04 μM peroxidase. D: 0.04 μM peroxidase+2 μM Cu++. E: 0.04 μM peroxidase+0.1 m*M* Mn++.

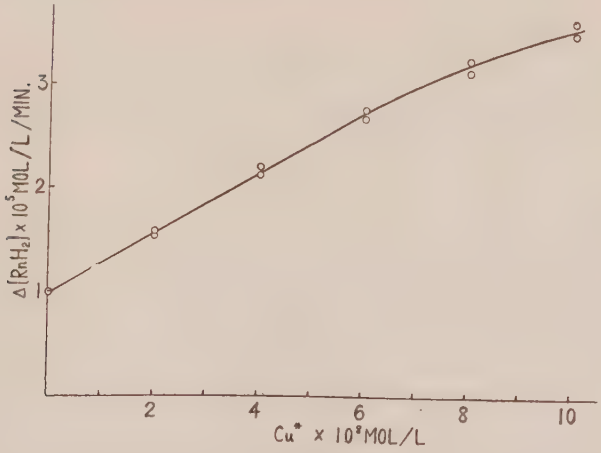

FIG. 6. Effect of Cu++ concentrations on the activation of RnH_2 oxidation in neutral pH.

0.1 μM peroxidase; 0.1 m*M* RnH_2. 2.5 m*M* phosphate buffer. pH 7.3. temperature 25°.

FIG. 7. Effects of Mn++, Cu++ and pH on DHM oxidation rate in 0.01 *M* acetate.

0.4 m*M* DHM, room temperature. △: autoxidation at pH 4.0, Mn++ free or 20 μM Mn++. □: 0.02 μM peroxidase, pH 4.0. ○: 0.1 μM peroxidase, pH 4.0. ×: 0.1 μM peroxidase, 0.033 μM Cu++, pH 4.0. ■: 0.02 μM peroxidase, 20 μM Mn++, pH 4.0. ●: 0.1 μM peroxidase, pH 5.3. ◒: 0.1 μM peroxidase, pH 3.5.

by the inhibitory action of catalase and some reducing agents. Very small amount of H_2O_2, which has been accumulated by the autoxidation of RnH_2, may act as a sparker of the reactions. The presence of such H_2O_2 is not detectable by the iodometric method, but confirmed by the spectroscopic observation as shown in Fig. 8. In the 0.25 μM peroxidase concentrations, no H_2O_2 accumulation is detectable and optical density of peroxidase at 420 mμ in the steady state is almost the same extent as that of peroxidase itself. However, the instantaneous increment of optical density at 420 mμ can be observed as soon as RnH_2 solution is added to the peroxidase solution. This suggests that complex II is formed in the presence of a trace amount of H_2O_2 ,which has been formed in the freshly prepared RnH_2 solution.

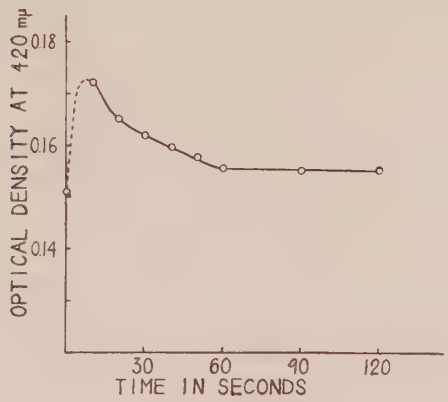

FIG. 8. Change of the optical density at 420 mμ on the addition of freshly prepared RnH_2 solution to the peroxidase solution.

0.25 μM peroxidase, 0.01 M acetate (pH 5.3), room temperature. Cell length: 100 mm.

According to the results shown in Fig. 2, H_2O_2 accumulation in the steady state of the reaction decreases as the peroxidase concentration increases and no more increment of the reaction rate is found above 0.1 μM peroxidase. This is explained by assuming that the increase of peroxidase concentration accompanies much smaller increment of the oxidative reaction rate than that of the peroxidative one. Further detailed analysis of the reaction mechanism by the usual kinetic method is quite difficult since the oxidative reaction rate depends upon the concentrations of H_2O_2, RnH_2, O_2, and peroxidase, and generally the amount of H_2O_2 is not maintained at a constant level during the reaction process.

In the concentration shown in Table I, metals such as Mn^{++}, Co^{++}, Hg^{++}, and Cu^{++} have no effects on the peroxidative Reaction (2) under anaerobic conditions. These metals exert the influences only on the oxidative reaction, but it is not yet clear whether these metals interact with peroxidase itself or with intermediate substances in the reaction.

Further investigations are now proceeding in our laboratory on the aerobic oxidase reaction of peroxidase from another point of view.

SUMMARY

Aerobic oxidation of triose reductone is catalyzed by the crystalline

turnip peroxidase. The reaction is composed of the two successive reactions similarly as reported by Chance with aerobic oxidation of dihydroxymaleic acid by the horse-radish peroxidase.

The presence of a trace amount of H_2O_2 is indispensable to the first oxidative reaction and Mn^{++} does not play any essential role in this reaction.

Other heavy metals such as Cu^{++} and Hg^{++} have a very interesting effect on the reaction.

Our thanks are due to Prof. Takasugi for generous advices.

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STUDIES ON NITROREDUCTASE OF MAMMALIA

I. NITRO-REDUCING CAPACITY OF THE LIVER AND ITS RELATION TO DEHYDROGENASE SYSTEMS

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INTRODUCTION

In the recent years, studies have been made on "nitroreductase" of bacteria and its action mechanism has been partially clarified (*1–7*). Animal tissues also have been shown to be capable of reducing nitro groups of some aromatic compounds: Bray *et al.* (*8*), studying the metabolism of nitrobenzoic acid in the rabbits, observed that 11–12 per cent of the dose was reduced to the corresponding aminobenzoic acid; Westfall (*9*) demonstrated the reduction of T.N.T. by rabbit tissue; and Parker (*10*) studied the reduction of the nitro group in 2,4-dinitrophenol by rat-liver homogenate, using malate and succinate as accelerating agents, and suggested that the nitro-reduction was coupled with the dehydrogenase systems. However, there has been no report on the existence of "nitroreductase" and its enzymatic mechanism in animal tissues.

The authors (*11*), during the previous studies on the detoxication of *p*-nitrophenol, confirmed that *p*-nitrophenol, when injected intraperitoneally, was partially (20–30 per cent) converted to substances which did not turn yellow in alkaline solution even after hydrolysis (*p*-nitrophenol characteristically shows yellow coloration in alkali), and it was suggested that this might be due to the reduction of the nitro group of the phenol, to *p*-aminophenol.

In the present series of experiments this possibility was confirmed and some evidences of the existence of "nitroreductase" in the liver and its relation to some dehydrogenases are described in this paper, its possible physiological rôle is also discussed.

EXPERIMENTAL AND METHOD

Estimation of Enzyme Activity—Both the amounts of remaning *p*-nitrophenol and *p*-aminophenol formed were estimated after the incubation of *p*-nitrophenol with the homogenates and other supplements.

Disappearance of *p*-nitrophenol was determined from the difference of color development (in 0.5 *N* NaOH at 420 mμ) between the controls (into which the enzyme was added just after the reaction was stopped) and the main experiments.

p-Aminophenol formed was indentified by paper chromatography and its quantity determined by its specific oxidative coupling reaction with phenol (indophenol reaction).

Paper Chromatography of p-Aminophenol—A mixture of *p*-nitrophenol and homogenate was incubated on a large scale (10-fold) for 1 hour. After the reaction was stopped with the addition of an equal volume of 2 *N* HCl, the reaction mixture was filtered and the filtrate was extracted with purified, peroxide-free ether for 6 hours to remove the remaining *p*-nitrophenol. The acidic solution was neutralized to pH 7.0 with solid Na_2CO_3, and then further extracted for 12 hours. The ether was evaporated and the second extract was applied on a paper using acetic acid, butanol, and water (1:4:2) as the developer. *p*-Aminophenol gave the same Rf value (6.4) as that of the control. Under the above conditions, no other spot was detected on spraying the Folin's reagent.

Estimation of p-Aminophenol by the Indophenol Reaction—Considerably large differences (17–18 per cent) were always observed between the nitrophenol disappearances and aminophenol formations estimated respectively by the methods described below. These differences may be due to either the adsorption of *p*-aminophenol onto the sediment during the deproteinization procedure, or the disappearance of *p*-aminophenol as well as that of the possible labile intermediates during the analytical procedure. No attempt was made to investigate these possibilities at the present, and both the amount of remaining *p*-nitrophenol and *p*-aminophenol formed were estimated to represent the nitro-reducing activities of the various tissue preparations in the following experiments.

Analytical Methods—unless otherwise noted all the experiments were carried out as described below and, if needed, the volume of the buffer was reduced to bring the total volume of the incubation mixture to 8 ml.: 3 ml. enzyme preparation, 2 ml. 10^{-3} *M* *p*-nitrophenol, and 3 ml. *N*/10 phosphate buffer, pH 6.8, were incubated for 1 hour at 37°.

After the incubation period, deproteinization was carried out by the addition of an equal volume of 10 per cent trichloroacetic acid, followed by filtration, and neutralization with *N* NaOH before the estimation of the phenols.

To 5 ml. of the neutralized filtrate, was added 5 ml. *N* NaOH, and the alkaline solution was ready for the photometrical determination of *p*-nitrophenol (using an electrophotometer; 420 mμ).

Another 5 ml. of the neutral solution was submitted to the estimation of *p*-amino-

phenol by the indophenol reaction; by the addition of 2.5 ml. N Na_2CO_3, 0.5 ml. 5 per cent phenol, and 2 ml. 0.2 per cent sodium ferricyanide with the vigorous shakings after each addition. The color intensity was measured at 640 mμ.

Tissue Homogenate—Fresh tissue was rapidly homogenized with $N/10$ phosphate buffer, pH 6.8 and centrifuged for 15 min., $600 \times g$. The supernatant was used as the homogenate.

Alcohol Dehydrogenase—Alcohol dehydrogenase was prepared from baker's yeast by the method of Racker (*12*).

Succinic Oxidase—Succinic oxidase was prepared from the pig heart muscle (13).

RESULTS

Reduction of p-Nitrophenol by Rat Tissues

TABLE I

Reduction of p-Nitrophenol by Rat Tissues

Organs	Disappearance of *p*-nitrophenol	Formation of *p*-aminophenol
Liver	0.0751 μM	0.0733 μM
Kidney	0.0296	0.0224
Heart	0.0224	0.0124
Muscle	0.0183	0.0095
Lung	0.0112	0.0033
Testicles	0.0096	0.0099
Spleen	0.0032	0.0033
Uterus	0	0
Vagina	0	0
Ovary	0	0

3 ml. of tissue homogenate was incubated respectively with 2×10^{-6} M *p*-nitrophenol and 0.1 N Phosphate buffer (total volume, 8 ml.) for 2 hrs. at 37°.

Reduction of p-Nitrophenol by Rat Liver Homogenate—Disappearance of *p*-nitrophenol by rat liver homogenate is shown in Figs. 1, 2, and 3.

Effect of Enzyme Inhibitors—Potassium cyanide, sodium azide, thiourea, and monoiodoacetic acid were respectively added to the incubation mixtures, and all were found to inhibit the reduction of *p*-nitrophenol.

Other Factors Affecting the Nitro-reducing Activity of the Liver Homogenate—Results from the inhibition experiments suggest the contribution of

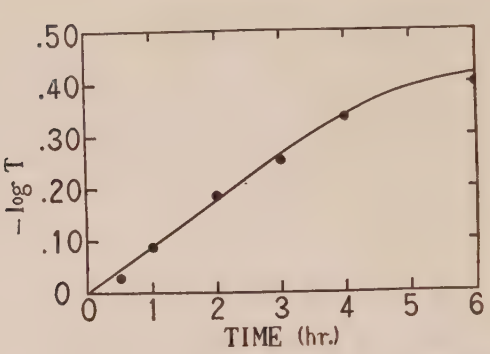


FIG. 1. Decoloration of *p*-nitrophenol by rat liver homogenate. a) Time-Decloration.

Four flasks containing 2 ml. 10^{-3} *M* *p*-nitrophenol, 3 ml. liver homogenate and 3 ml. *N*/10 phosphate buffer were incubated for 0.5, 1, 2, 3, 4 and 6 hrs. at 37°. Homogenate: Five grams of the liver was homogenized in 25 ml. *N*/10 phosphate buffer, pH 6.8, and centrifuged for 10 min., 600 *g*. The supernatant was offered as homogenate. (Unless otherwise noted, homogenate was prepared with this method in the experiments described in this paper).

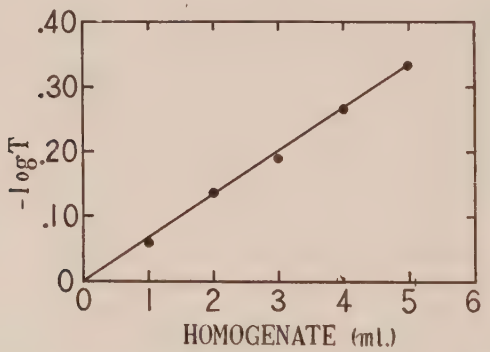


FIG. 2. Decoloration of *p*-nitrophenol by rat liver homogenate. b) Enzyme concentration-decoloration.

Incubation flasks contained 2 ml. 10^{-3} *M* nitrophenol, 3 ml. *N*/10 phosphate buffer, and 1, 2, 3, 4 or 5 ml. of liver homogenate. The mixture was incubated for 2 hrs. at 37°.

some heavy metals to the nitro-reducing reaction, which possibly con-

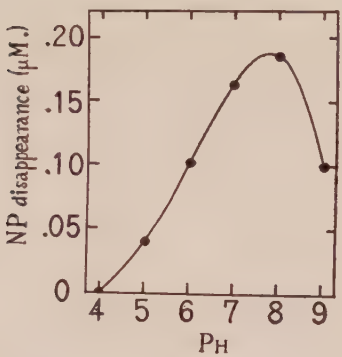


FIG. 3. pH relation of the disappearance of *p*-nitrophenol.

Incubation flasks contained 2 ml. 10^{-3} *M* *p*-nitrophenol, 3 ml. liver homogenate, and 3 ml. *N*/10 phosphate solution at pH 4, 5, 6, 7, 8, or 9 (pH was adjusted with HCl or NaOH). The amount of *p*-nitrophenol disappeared (μM per ml. homogenate) was dotted on the ordinate.

TABLE II

Inhibitors

Inhibitors	Concentration	Inhibition	
		p-Aminophenol formation	*p*-Nitrophenol reduction
	M	%	%
Potassium cyanide	10^{-6}	100	100
Sodium azide	3×10^{-3}	70	50
Thiourea	2×10^{-3}	93	80
Monoiodoacetic acid	10^{-2}	100	100

sists in the hydrogen transfer reaction of some dehydrogenase systems in a similar manner to that of the nitrate reductase of plants and bacteria (*15*). In that case, it will be expected that the reduction reaction will proceed even in anaerobic conditions. These possibilities were examined and the nitro-reduction was proved to proceed both *in vacuo* and in nitrogen gas, as fast as, or somewhat faster than, in the air.

Parker (*10*) confirmed that substrates of dehydrogenases (lactate and succinate he tested) increased the rate of reduction of one nitro

TABLE III

p-Nitrophenol Reduction under Anaerobic Conditions

	p-Nitrophenol disappearance	*p*-Aminophenol formation
In air	9.88 μg.	6.08 μg.
In vacuo	11.00	6.44
In N_2	10.10	—

group of 2,4-dinitrophenol by the liver homogenate. Succinate, glucose and alcohol were applied to our rat liver homogenate, but the reduction of *p*-nitrophenol was found not to be affected by the addition of these dehydrogenase substrates.

Effect of Dialysis and Its Restoration—To investigate the effect of co-factors, liver homogenate was dialyzed (6 hours against tap water at room temperature, and 15 hours against distilled water at 0°).

Dialysis removed the nitro-reducing capacity of the homogenate almost completely and the addition of a boiled juice of the liver was proved to restore the activity entirely.

According to Saz and Slie (*3*), the "nitroreductase" of bacteria is coupled with the dehydrogenases *via* DPN or TPN. Assuming that the mechanism of nitro-reduction of the liver is similar to that of bacteria, the restoring action of the boiled juice may suggest that the dialysis of the homogenate results in the removal of the substrates and/or the coenzymes of the dehydrogenases.

The effect of succinate, alcohol, and glucose were examined, and it was shown that glucose or alcohol with DPN, or succinate alone were able to restore the reducing activity of the dialysate, suggesting that succinic, alcohol, and glucose dehydrogenases were responsible, perhaps indirectly, for the reduction of *p*-nitrophenol.

Comparison of the Nitro-reducing Activity and Succinic Dehydrogenase Activity of Some Tissues of the Rat—This experiment was carried out in order to confirm the existence of an enzyme which is directly responsible for the reduction of the nitro group.

Greville and Stern (*14*) showed that a bacterial succinic dehydrogenase reduces a nitro group of 2,4-dinitrophenol. If it be true of the nitro-reducing capacity of the liver homogenate mentioned above, that is, if the nitro group could be reduced without any specific enzyme directly taking a part, the nitro-reducing capacity should roughly cor-

TABLE IV

Restoration of the Activity in Dialysate

	p-Nitrophenol disappearance	*p*-Aminophenol formation
	μM	μM
Dialysate	0	0
,, + DPN	0	0
,, + glucose	0	0
,, + alcohol	0	0
,, + glucose + DPN	0.0641	0.0635
,, + alcohol + DPN	0.0539	0.0520
,, + succinate	0.0646	0.0628
,, + boiled juice	0.0695	0.0598

Glucose: 2.5×10^{-4} *M*. Alcohol: 10^{-3} *M*. Succinate: 2.5×10^{-4} *M*.

respond proportionally to the succinic dehydrogenase activity.

Obviously, the present results show the different distribution between these two activities and suggest the existence, besides the succinic dehydrogenase, of an indespensable enzyme or enzyme system for the nitro-reduction, which may be named "mammalian nitroreductase."

TABLE V

Nitro-reduction and Succinic Dehydrogenase Activity of Tissues

Organs	*p*-Nitrophenol disappeard	Succinate oxidation	
		O_2-uptake*	Methylene blue decoloration**
	μM	$\mu l.$	*min.*
Liver	0.0751	87.4	6.00
Kidney	0.0296	442.4	5.00
Heart	0.0224	715.9	4.20
Muscle	0.0183	100.9	18.10

* O_2 μl./mg. (wet itssue)/hr.
Succinate: 2.5×10^{-4} *M*. pH 6.8. 30°.
** 2.5×10^{-4} *M* succinate, 30°, pH 6.8.

Nitro-reducing Activity of Pig Liver, and Its Acetone Powder—Pig liver homogenate was proved to be 6–7 times more active than that of a rat, the activity of which was removable by the dialysis and also restorable

by the addition of glucose or alcohol plus DPN, and used as an enzyme source in the fractionation experiment described below.

The acetone powder was prepared as usual from a pig liver, and shown, similary to the dialysate, to be incapable of reducing *p*-nitrophenol and to be restored by the addition of glucose and alcohol plus DPN.

TABLE VI

Restoration of the Activity in Acetone Powder

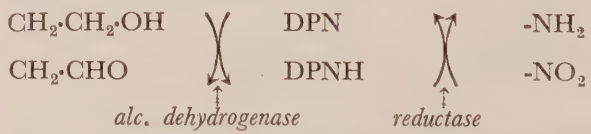
	p-Nitrophenol disappearance	*p*-Aminophenol formation
	μM	μM
Acetone powder	0	0
,, + DPN	0	0
,, + glucose	0	0
,, + alcohol	0	0
,, + glucose + DPN	0.1508	0.1307
,, + alcohol + DPN	0.1769	0.1543
,, + boiled juce	0.0642	0.0631

An adequate amount of dried acetone precipitate was ground with the buffer, and the homogenized solution was submitted to the test. Concentrations of the supplements were similar to those listed in Table IV.

Fractionation of the Homogenate—Pig liver homogenate was devided into two fractions, one that precipitates in 0–33 per cent acetone (*Powder I*) and the other in 33–50 per cent acetone (*Powder II*). They were examined separately with alcohol dehydrogenase system.

DISCUSSION

From the results listed in Table VII, it is clear that the "nitroreductase" is quite different from alcohol dehydrogenase and the H atom derived from alcohol is transferred to the nitro group *via* DPN only by the action of "nitroreductase."



Succinic oxidase, from a pig heart muscle, failed to reduce *p*-nitrophenol, indicating that the "nitroreductase" also differs from succinic

TABLE VII

Fractionation with Acetone

Addition	*p*-Nitrophenol disappearance
	μM
Powder I	0
,, + alcohol	0
,, + ,, + DPN	0.045
,, + ,, + ,, + alcohol dehydrogenase	0.194
Powder II	0
,, + alcohol	0
,, + ,, + DPN	0.158
,, + ,, + ,, + alcohol dehydrogenase	0.183
Aochol dehydrogenase + alcohol + DPN	0

Alchol dehydrogenase: 5 mg. The concentrations of the other supplements were similar to Table IV.

dehydrogenase. Greville and Stern's data (*14*), which showed the capacity of bacterial succinic dehydrogenase to reduce the nitro group of dinitrophenol, should be reinvestigated. Though, from the dialysis experiment (Table IV), the coupling capacity of nitroreductase with succinic dehydrogenase is undoubted, the restoration of the activity in the acetone powder by the addition of succinate or succinate plus succinic oxidase was scarcely found, and the coupling mechanism of succinic dehydrogenase was left for later examinations.

Parker's (*10*) data, which have shown the acelerating effect of succinate or lactate on nitro-reducing action of liver homogenate, are reasonable. When referred to the effect of succinate on our dialysate, and the reverse results in this paper in the homogenate experiment, the failure of succinate and glucose plus DPN to show the acceleration, may be due to the sufficient amount of these dehydrogenase substrates remaining in the homogenate.

It is supposed that almost all of the dehydrogenase systems are coupled to the nitroreductase systems *via* DPN or TPN, and in the organs, like the liver, in which the substances are being rapidly metabolized, the "nitroreductase" is acting as a DPN-oxidizing enzyme, so that the metabolism should be smoothly carried out, even in the absence of oxygen, by the action of reductase in place of the cytochrome system.

SUMMARY

1. The nitro-reducing capacity of the mammalia *in vitro* was discussed.

2. The liver, kidneys, heart, muscle, lung, testicles, spleen, uterus, vagina, and ovary of rats were homogenized and examined, and the former seven were found able to reduce *p*-nitrophenol in the above order.

3. The reduction of the nitro group by the liver is inhibited by cyanide, monoiodoacetic acid, sodium azide, and thiourea, suggesting the indispensable participation of heavy metals in the reduction mechanism.

4. Dialysis removed the reducing capacity of the liver, which is restored by the addition of a boiled juice of the liver, succinate, glucose plus DPN, and alcohol plus DPN.

5. Acetone precipitation also removed the capacity, which was restored by the addition of the boiled juice, glucose and alcohol with DPN.

6. The acetone fractionation of the liver homogenate affords a fraction which possesses nitro-reducing capacity only when coupled with alcohol dehydrogenase system (alcohol plus alcohol dehydrogenase plus DPN). The alcohol dehydrogenase system failed to reduce *p*-nitrophenol in the absence of the nitroreductase fraction of the liver.

7. The difference in the order of the distribution between the nitro-reducing capacity and succinic dehydrogenase activity was shown and the existence of an enzyme was discussed which plays an indispensable role directly in the reduction of the aromatic nitro group in the mammalia. This enzyme may be termed a "mammalian nitroreductase."

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STUDIES ON THE ABSORPTION OF UNNATURAL MONOSACCHARIDES AND THEIR FATES AFTER BEING ABSORBED

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The well known phosphorylation theory about the absorption of natural sugars from intestine was first set forth by Wilbrandt (*1*), Verzár (*2*), and their co-workers. An important support has recently been given to the theory in our laboratory by Ota and Shibata (*3*) who clarified the mechanism of sugar absorption by the use of P^{32}.

But little is known about the absorption of the optical antipode of the natural monosaccharide, and still less about their metabolism. Rudney (*4*) reported that L-glucose was utilized neither in a rat nor in any bacteria and that it was eliminated in urine in a rat. For arabinose, Saundars (*5*) demonstrated in his experiments that L-arabinose was fermented readily, while D-arabinose with difficulty by many types of bacteria except three strains of the Proteus vulgaris. Neuberg and Wohlgemuth (*6*) also stated in their experiments using rabbits that L-arabinose was concerned with the glycogen formation in liver, but D-arabinose did not and that L-arabinose resembled D-glucose more closely than D-arabinose did in the manner they were metabolized. Ota's experiment (*7*) using mice agreed with Neuberg and Wohlgemuth's, in result. However, Corley (*8*) stated that L-and D-forms of arabinose were metabolized with about equal facility in rabbit tissue, but that if there is any difference of utilization, D-form seems more easily metabolized. Roe (*9*) also stated that D-arabinose formed glycogen though in an insignificant quantity and underwent a metabolic transformation. Thus the metabolisms of these sugars still reserve an interesting problem waiting for further investigation, since no definite result has been obtained.

The problems to be taken up in the present study are the absorption of unnatural monosaccharides in its relation to the phosphorylation in intestinal mucosa using P^{32} and the formation of glycogen in the liver.

EXPERIMENTAL

Materials

D-Glucose, L-galactose, D-galactose, L-mannose and D-mannose were obtained from the commercial sources. L-Glucose was prepared by reduction of L-gluconic acid according to Steiger (*10*) and the latter was obtained from L-arabinose by the method of Hudson (*11*). D-Arabinose was prepared from calcium gluconate by the method of Hockett and Hudson (*12*). L-Arabinose was obtained from arabic gum. Each sugar with the exception of L-mannose was checked as to its purity by its melting point and specific rotation.

The melting points and the specific rotations of monosaccharides.

	m.p.	$[\alpha]_{20}^{D}$		m.p.	$[\alpha]_{20}^{D}$
L-Glucose	144.0	−51.3	D-Glucose	146.0	+52.3
L-Galactose (from General Biochem. Inc.)	162.0	−80.7	D-Galactose	161.5	+80.5
L-Mannose (syrup) (from General Biochem. Inc.)			D-Mannose (syrup)		
L-Arabinose	158.5	+102.0	D-Arabinose	159.0	−103.0

Perfusion Experiment

A group of two to five young rabbits weighing from 600 to 800 g. was used in each experiment. After fasting for 24 hours, the abdomen was cut open without anesthesia and the intestinal segment located 30 to 60 cm. below the stomach was removed for the perfusion experiment. A perfusion solution was prepared according to Krebs and Henseleit (*13*), but deprived of its calcium content in order to prevent the contraction of the intestine during the perfusion. In the absorption test was used Shibata's modification (*3*) of Darlington and Quastel's apparatus (*14*). Various sugars (0.45 per cent in final concentration) and $NaH_2P^{32}O_4$ (1.45×10^6 c.p.m.) were added simultaneously to the inner circulating fluid.

Determination of Absorption Rates of Various Sugars—Samples were removed by pipetting from the outer circulating fluid at intervals of 20 minutes for 80 minutes. The amount of absorbed sugar was determined by the method of Hagedorn-Jensen (*15*).

Static and Dynamic Changes of Phosphates in the Intestinal Mucosa—At the end of the perfusion experiment, the intestinal mucosa was scratched off as quickly as possible and fractionated into the five phosphate fractions of total P, acid soluble P, barium soluble P, inorganic P and the easily hydrolysable phosphate portion of ATP (ATP-10′-P). The ATP-10′-P fraction was separated by Lipmann's method (*16*), barium soluble P by LePage's (*17*). The samples thus prepared were ashed with perchloric acid and their phosphorus contents were estimated by the method of Barton (*18*). The phosphate in each fraction was then precipitated to form $Mg(NH_4)PO_4$ by the

addition of the carrier and its P^{32} content was assayed with an endwindow Geiger-Mueller tube. The value for the specific activity of the phosphate in each fraction was expressed in terms of counts per minute of P^{32} per mg. of P^{31}. The counting rate of each sample was corrected for decay. The dynamic changes of phosphates in the intestinal mucosa during the absorption of the various sugars were investigated in comparison with the specific activities of these phosphate fractions.

EXPERIMENT IN SITU

Male and female albino rats weighing from 200 to 280 g. were used. After fasting for 48 hours, the rat was anesthesized slightly with ether, a laparotomy was performed and the ligature was placed around the esophagus. A sugar solution was injected directly into the stomach. The abdomen was closed. About 10 ml. of 0.85 per cent NaCl solution was injected subcutaneously and the urethra was ligated in order to collect the urine quantitatively. The rat was sacrificed at the 4th hour after the sugar administration, 0.1 ml. of blood collected from Vena cava caudalis for determination of the blood sugar level, and the liver removed and weighed as quickly as possible for determination of its glycogen content by the method of Somogyi (*19*). The stomach, the small intestine and the whole large intestine were carefully detached from the mesentery. The whole intestinal tract was cut open and washed with distilled water. The sugar contents of the intestinal tract and the urine in the bladder were determined by the method of Hagedorn-Jensen. The dosage of sugar used in the experiment was 250 mg. per 100 g. of body weight.

RESULT AND DISCUSSION

The Velocity of Sugar Absorption—As illustrated in Fig. 1, the amount of sugar coming into the outer circulating fluid by diffusion and absorption increased linearly for 80 minutes, and both enantiomorphs of each sugar examined were absorbed almost at the same rate. The concentrations of the sugars came into the outer circulating fluid in 80 minutes were as follows:

L-Glucose	11.0	mg.%	D-Glucose	11.4	mg.%
L-Galactose	12.1	,,	D-Galactose	12.5	,,
L-Mannose	5.8	,,	D-Mannose	5.7	,,
L-Arabinose	3.7	,,	D-Arabinose	3.8	,,

The absorption rate of glucose and arabinose in rat intestine *in situ* is indicated in Table II.

The difference in absorption rate of D- and L- forms of each sugar, in particular of the latter, is very small and may be considered to have confirmed the results of the perfusion experiments above described.

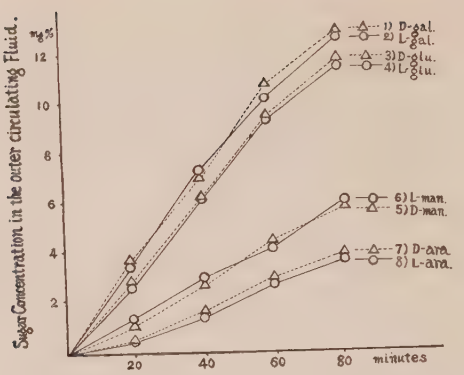

FIG. 1. Graphic representation of absorption rates of various sugars.

1) D-Galactose (Average of 2 expt.)
2) L-Galactose (Average of 2 expt.)
3) D-Glucose (Average of 5 expt.)
4) L-Glucose (Average of 5 expt.)
5) D-Mannose (Average of 2 expt.)
6) L-Mannose (Average of 2 expt.)
7) D-Arabinose (Average of 5 expt.)
8) L-Arabinose (Average of 5 expt.)

The Changes of Phosphates during Sugar Absorption—It is shown in Table I that the specific activities of each phosphate fraction in the intestinal mucosa during absorption of L-form of various sugars employed have nearly the same intensity as those of their optical antipodes. The increases in the specific activity of acid soluble-, ATP-10′- and barium soluble P were almost the same in both antipodes. Only in arabinose the increase was not remarkable, although even in this case the difference of specific activity between both enantiomorphs was scarcely to recognize. The above findings lead naturally to the conclusion that phosphorylation takes place on both antipodes of sugar in the same grade during their absorption from the intestine.

The Metabolism of Sugar—The blood sugar level was 236 and 190 mg. per cent at 4th hour after the application of L- and D-glucose *in situ* respectively as shown in Table II. Moreover a higher level has been maintained for long time by L-glucose, viz. 225 mg. at 6th hour after administration.

The difference can be interpreted as a result of remaining of L-glucose in rat body longer than D-glucose without being metabolized.

Both forms of arabinose behaved almost like those of glucose, though he difference between them in this respect was not so significant because

TABLE I

Changes of the Phosphate Fractions during the Absorption of Various Sugars

(c.p.m.)

Sugar		*Inn. sol.	†Outer solution			Intestinal mucosa				
		Total P	Total P	Inorganic P	Inorg. P. / Total P	Total P	Acid. soluble P	Inorganic P	ATP-10′-P	Barium soluble P
‡Control P^{32} only		10929	1133	1095	96.7%	2276	8091	14900	8854	3770
Glucose	L	9985	3527	3488	98.8	5272	12184	17587	20315	7015
	D	11031	3558	3554	99.8	5521	14894	19504	21601	7181
Galactose	L	11040	4084	4096	100.2	6758	16992	21616	26856	9136
	D	10123	4054	4101	101.1	6888	17572	22212	27784	9216
Mannose	L	10034	2724	2695	98.9	3724	9781	12422	18507	5264
	D	10125	2740	2728	99.5	3740	9704	13154	19165	5118
Arabinose	L	9851	1998	2045	102.3	2944	8475	10852	16703	3487
	D	10423	2033	1998	98.3	3067	9196	11190	16741	3721

* Radioactivity of 1 ml. of inner solution.

† Radioactivity of 10 ml. of outer solution.

The numbers show the specific activity of each phosphate fraction.

‡ Control is quoted from Shibata's report (*3*).

TABLE

The Absorption Rate, Blood Sugar Level, Liver Glycogen and Sugar Content Excreted

(Dosage 250 mg. per

	Body weight			Blood sugar
	Before fasting	After fasting for 48 hrs.	Loss of body weight	
	g.	*g.*	*%*	*mg./dl.*
Non-fasting rat				
	230	230	0	121
	220	220	0	124
Average	225	225	0	122
Control (0.85% NaCl solution)	230	205	10.9	96
	240	210	12.5	86
Average	235	207	11.7	92
L-Glucose				
	260	230	11.5	223
	210	180	11.9	226
	280	240	14.2	259
Average	253	218	12.5	236
D-Glucose				
	210	185	11.9	174
	180	155	13.8	186
	260	255	13.4	205
	220	190	13.6	196
Average	217	188	13.1	190
L-Arabinose				
	220	190	13.6	201
	220	185	15.9	214
	250	220	12.4	195
Average	230	198	13.9	203
D-Arabinose				
	210	180	14.2	213
	230	205	10.8	235
	230	200	13.0	229
Average	223	195	12.6	225

of the difficulty with which arabinose was utilized even in natural form.

The glycogen content in liver amounted to 80.5 and 9.0 mg. per cent respectively in 4 hours after the administration of D- and L-glucose. Even the quantity of the former was smaller than the corresponding value in non-fasting rats, presumably because of depleted glycogen store and the surgical procedures. The latter value approached to the control which had been given NaCl solution by injection. These results

II

in the Urine at 4th Hour after the Administration of Various Sugars to Rats

100 g. of body weight)

Liver glycogen		Absorption		Urine sugar
Liver weight	Glycogen content	Sugar content in intestine	Rate of absorption	
g.	*mg.*	*g.*	*%*	*mg.*
5.4	3100			
5.2	2400			
5.3	2750			
4.7	8.8	4.5		
5.1	10.2	3.7		
4.9	9.5	4.1		
5.5	8.2	90.8	84.2	30.4
4.6	10.5	62.4	86.5	39.6
5.7	8.5	94.2	84.3	33.9
5.5	9.0	82.4	85.0	34.6
5.1	82.1	48.5	89.5	
4.4	65.5	38.4	80.1	28.1
4.7	105.7	75.4	86.6	17.6
4.8	69.0	64.1	86.5	13.2
4.0	80.5	56.6	88.1	19.6
4.8	13.2	282.6	40.5	43.1
4.6	14.8	280.2	39.4	32.7
5.5	11.2	303.1	44.9	69.5
5.0	12.7	288.6	41.6	48.4
4.6	8.5	279.4	37.9	38.4
5.0	9.1	273.1	46.7	35.6
4.7	9.2	320.5	35.9	28.9
4.8	8.9	291.0	40.2	34.3

obtained indicate that L-glucose was not concerned with the glycogen formation in liver.

The glycogen content in liver after the application of both forms of arabinose was similar as in the case of glucose in some extent, though glycogen was formed in a smaller amount even after administration of the natural form, which showed much less utilization of it.

As shown in Table III, the total urinary elimination of sugar after

administration of D- and L-glucose was 19.6 mg. and 34.6 mg. in 4 hours, 20.4 mg. and 69.5 mg. in 6 hours respectively.

It can be considered that the rate of disposal of L-glucose from the tissue is not parallel to the rate of its absorption and that almost quantity of sugar injected is eliminated in the urine, L-glucose being excreted in a longer time than D-glucose and keeping up the sugar content of blood at a higher level, *viz.* at 225 mg. per cent at the 6th hour than the latter (Table III).

TABLE III

Urine and Blood Sugar (mg./dl.)

	2 hrs.		4 hrs.		6 hrs.	
	Urine	Blood	Urine	Blood	Urine	Blood
D-Glucose	13.5	212	19.6	190	20.4	135
L-Glucose	16.2	208	34.6	233	69.5	225
D-Arabinose	10.6	184	34.3	226	62.0	246
L-Arabinose	12.7	171	48.4	203	51.7	210

The amount of L- and D-arabinose eliminated in the urine in 6 hours was 51.7 and 62.0 mg. respectively, showing the metabolic difficulty of both sugars. The fact that L-arabinose was found in a smaller quantity than D-arabinose in blood and urine at the 6th hour (Table III) and that the measure of glycogen formed in liver (Table II) after administration of L-arabinose was greater than after that of D-arabinose, indicate that L-arabinose is metabolized to a small extent in the rat body, while D-enantiomorph is all eliminated in the urine without utilization. The present conclusion agrees with Neuberg and Wohlgemuth's and Ota's.

SUMMARY

1. Both antipodes of glucose, galactose, mannose and arabinose were compared as to the absorption rate, glycogen formation in the liver, the blood sugar level and their elimination in the urine.

2. Both forms of these sugars examined were absorbed from the intestine increasing linearly for 80 minutes and no evidence has been obtained of any remarkable difference of the absorption rate between

the both antipodes of each sugar.

3. The selective absorption of the unnatural enantiomorphs of hexose and pentose so far examined seemed to have some relation to the phosphorylation in the intestinal mucosa.

4. The unnatural form of sugars studied was not concerned with the formation of liver glycogen and likely to be eliminated in the urine without utilization.

The author wishes to express his deep gratitude to Prof. Dr. R. Hirohata, director of the Department, for the carefull guidance given him throughout the present research.

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THE PRESENCE OF PYRIDINE IN LIPOPROTEIN OF BCG

Dear Sirs:

Lipoprotein or proteolipide is a subject of interest from the standpoint of the allergic antigen. Waksman, Porter, Lees, Adams and Folch (*1*) succeeded in the production of experimental allergic encephalomyelitis in rabbits with the proteolipide from cattle brain, and Yamamura, Yasaka, Nakamura, Yamaguchi, Ogawa, Endo, and Takeuchi (*2*) succeeded in the formation of tuberculous cavity in lungs of rabbits with the lipoprotein from BCG. Of the chemical structures of lipoprotein, however, little is known.

In the course of chemical study of the lipoprotein of BCG, we found some absorption bands (at about 246, 252, and 256 mμ) in the ultraviolet region of methanol-chloroform solution of the lipoprotein. In the paper chromatogram of acid hydrolysate of the lipoprotein, a spot was detected by ultraviolet photography. The substance was identified as pyridine from its volatility, Rf value and ultraviolet absorption spectrum. (Recently a substance supposed to be pyridine pentotide could be detected in the partial hydrolysate obtained under mild condition of the lipoprotein by paper chromatography. The presence of pyridine pentotide would exclude the possibility that pyridine is not a product of bacterial action.)

BCG was cultured on modified Sauton's media (asparagine was substituted for sodium glutamate) for 6 weeks. The harvested cells were dried with acetone and extracted with chloroform-methanol (1 : 1). From the extract the lipoprotein was prepared by the method of Folch, Ascoli, Lees, Meath and LeBaron (*3*) and hydrolyzed with 6 N HCl for 18 hours. The hydrolysate was concentrated on the water bath, and aliquots of concentrate and authentic specimens (dissolved in N HCl) were chromatographed with *n*-butanol-methanol-N HCl (20 : 3 : 3) as solvent system. The spots were detected by ultraviolet photography. The substance is considered to be a volatile weak base, since the spot could not be detected when the solvent systems containing no hydrochloric acid were used or when the paper chromatogram was heated at 80° for a few minutes. The spot to be identified was coinci-

dent with that of pyridine but neither with those of picolines nor of pyridine carboxylic acids (Table I). The absorption spectra of the eluates of the spots with *N* HCl also showed that the unknown substance was pyridine (Fig. 1).

TABLE I

Rf Values of Unknown Substance in the Acid Hydrolysate of Lipoprotein of BCG and Pyridine Derivatives

Paper: Toyo-Roshi No. 5 A, solvent: *n*-Butanol-methanol-*N* HCl (20:3:3), ascending method, 15 hours, detection by ultraviolet photography

Material	Rf
Unknown substance	0.20
Pyridine	0.20
Picolines (α, β, γ)	0.34
Picolinic acid	0.34
Nicotinic acid	0.28
Isonicotinic acid	0.31

The presence of pyridine in the hydrolysate of lipoprotein gives some suggestions on amino acid metabolism. Pyridine would be formed from nicotinic or picolinic acid. Although the derivatives of both of the acids are found in biological material, the precursor of pyridine would be picolinic acid, because picolinic acid is decarboxylated more easily than nicotinic acid by chemical method. Picolinic acid might be derived from diaminopimelic acid (*4*), lysine or hydroxylysine (*5*) which are found in lipid fractions of mycobacteria.

The chemical structure and properties of picolinic acid are similar to those of isonicotinic acid. We presume that isonicotinic acid hydrazide (INH) would be metabolic antagonist of picolinic acid. Barclay, Ebert and Koch-Weser (*6*) suggested that INH was fixed by the INH sensitive strain of tubercle bacilli and might block the formation of a substance essential for cell division. From these facts we now expect that INH will block the formation of lipoprotein by inhibiting a certain step of picolinic acid metabolism.

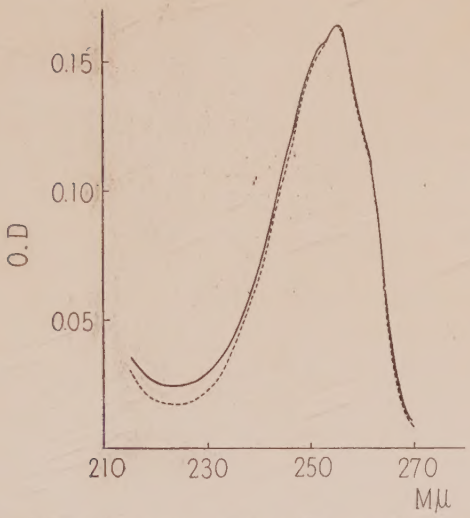

FIG. 1. Absorption spectra of the unknown substance in the acid hydrolysate of lipoprotein of BCG and of pyridine in N HCl eluted from paper chromatogram.

——— unknown substance in the acid hydrolysate of lipoprotein of BCG.

..... authentic pyridine.

(Beckman model DU quartz spectrophotometer).

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